Human skin equivalents to study the prevention and treatment of wound infections

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CHAPTER 3

LL-37-derived peptides eradicate multidrug-resistant Staphylococcus aureus from thermally wounded human skin equivalents

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Abstract

Burn wound infections are often difficult to treat due to the presence of multidrug-resistant bacterial strains and biofilms. Currently, mupirocin is used to eradicate methicillin-resistant *Staphylococcus aureus* (MRSA) from colonized persons; however, mupirocin resistance is also emerging. Since we consider antimicrobial peptides to be promising candidates for the development of novel anti-infective agents, we studied the antibacterial activities of a set of synthetic peptides against different strains of *S.aureus*, including mupirocin-resistant MRSA strains. The peptides were derived from P60.4Ac, a peptide based on the human cathelicidin LL-37. The results showed that peptide 10 (P10) was the only peptide more efficient than P60.4Ac, which is better than LL-37, in killing MRSA strain LUH14616. All three peptides displayed good antibiofilm activities. However, both P10 and P60.4Ac were more efficient than LL-37 in eliminating biofilm-associated bacteria. No toxic effects of these three peptides on human epidermal models were detected, as observed morphologically and by staining for mitochondrial activity. In addition, P60.4Ac and P10, but not LL-37, eradicated MRSA LUH14616 and the mupirocin-resistant MRSA strain LUH15051 from thermally wounded human skin equivalents (HSE). Interestingly, P60.4Ac and P10, but not mupirocin, eradicated LUH15051 from the HSEs. None of the peptides affected the excretion of interleukin 8 (IL-8) by thermally wounded HSEs upon MRSA exposure. In conclusion, the synthetic peptides P60.4Ac and P10 appear to be attractive candidates for the development of novel local therapies to treat patients with burn wounds infected with multidrug-resistant bacteria.

Introduction

Each year, about 11 million people worldwide seek medical treatment for severe burns, of whom 300,000 will die (1). In the past few decades, great progress has been made in the care and treatment of burn wound victims, but infections with, e.g., *Staphylococcus aureus* or *Pseudomonas aeruginosa* still cause serious morbidity and mortality (2–4). Uncontrolled wound infections may result in delayed wound healing and/or cause severe systemic infection in burn patients (5).

The emergence of antimicrobial resistance hampers both current strategies to prevent bacterial colonization of the wound bed and the treatment of infections that arise from colonization. For example, topical antibiotics, like mupirocin (6) and Neosporin (7), are ineffective when resistant bacteria colonize the wound (8, 9). Furthermore, these antimicrobials are not effective because bacteria in biofilms can be up to 1,000-fold less sensitive to antibiotics than their planktonic counterparts (10–12). Other topical disinfectants, such as chlorhexidine (13), silver sulfadiazine (14), and iodine preparations (15), can be very painful when applied to open wounds (16), and the scientific evidence for the efficacy of these agents in wounds is scarce. Clearly, there is an urgent need for novel antimicrobial agents that can be applied topically to (i) prevent colonization and (ii) eliminate infectious agents in burn wounds (17).

We consider antimicrobial peptides (AMPs) to be potential therapeutic compounds. AMPs are essential components of the human innate immune system and as such contribute to the first line of defense against infections (18, 19). The human cathelicidin hCAP-18 releases the active cationic peptide LL-37, which displays bactericidal and antibiofilm properties against Gram-positive *S. aureus* (20–22) and *P. aeruginosa* (23) and lipopolysaccharide (LPS) neutralization (24) and aids in wound healing (15, 25). The synthetic LL-37-derived peptide 60.4Ac (P60.4Ac) retains the -helical structure of the parent peptide (26) and displays enhanced antimicrobial properties against Gram-negative bacteria and fungi compared to those of LL-37. Moreover, P60.4Ac retains the LPS-neutralizing activities of LL-37 (26) and has proven to be beneficial in patients with otitis media (27, 28). LL-37 has been successful in enhancing wound healing in patients with chronic venous leg ulcers (in a clinical phase I/II study conducted by Pergamum) and in diabetic patients suffering from infected wounds (29).

We recently developed a thermally wounded skin infection model and demonstrated that methicillin-resistant *S. aureus* (MRSA)-infected human skin equivalents (HSEs) can be successfully treated using mupirocin (30). HSEs are

air-exposed threedimensional human skin models that mimic the native skin to a high degree. They can be used to study many properties of the human skin, including barrier properties (16) and wound healing (31). Furthermore, HSEs have been used to study skin colonization with MRSA (32, 33). In the present study, we exploited the thermally wounded HSE infection model to investigate the antimicrobial activities of a new set of synthetic peptides based on the sequence of P60.4Ac. The aim of this study was to compare the antibacterial activities of these novel synthetic AMPs against MRSA to those of the parent peptides P60.4Ac and LL-37, as well as to mupirocin.

Materials and methods

Antimicrobial agents

All peptides (N-terminal acetylated and C-terminal amidated) were synthesized by solid phase strategies on an automated multiple peptide synthesizer (SyroII, MultiSyntech, Witten, Germany) as described (26). A set of 14 peptides was designed by substitution of one or more amino acids in the sequence of P60.4Ac in such a way that the alpha-helix was predicted to be retained. The sequences of the peptides in this study are shown in Table 1. The molecular mass of the peptides was confirmed by mass spectrometry. The purity of the peptides was >90%, as determined by reverse-phase high performance liquid chromatography; peptides LL-37, P60.4Ac and Peptide 10 (P10) in the experiments were > 95% pure. The lyophilized peptides were stored at -20°C until use. For experiments peptides were dissolved in H_2O with 0.01% (v/v) acetic acid to a stock of 10 mM, aliquots were made and stored at -20°C. Prior to the experiments, peptide-stocks were further diluted in phosphate buffered saline (PBS; pH 7.4). Mupirocin was obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands) and dissolved in PBS.

Bacteria

The following *S. aureus* strains were used in this study: LUH14616 (sequence type 247), LUH15051 (sequence type [ST239]), Saco042, a USA 300 strain (ST8), Imp126 (ST121) and NTCT 8325-4 (ST8). LUH14616, a clinical MRSA isolate was kindly provided by dr. S. Croes (34), LUH15051, a mupirocin resistant clinical isolate, was kindly provided by Dr. M. Heck (Laboratory of Infectious Diseases and Screening, National Institute for Public Health and Environment, Bilthoven, The Netherlands). The other strains were a kind gift from dr. WJB van Wamel (Dept of medical microbiology and infection, Erasmus medical center, Rotterdam, The Netherlands. All strains were typed using multi locus sequence

typing (35). Bacteria were stored in glycerol at -80°C until use. Prior to experiments, bacteria were subcultured on sheep blood agar plates (Biomerieux, Marcy l'Etoile, France).

In vitro killing assay

Bacteria were cultured to mid-log phase in Tryptic Soy Broth (TSB) (Oxoid Limited, Basingstoke, United Kingdom) for 3 hrs at 37°C under continuous rotation (200 rpm). Hereafter, bacteria were centrifuged at 1,000 x *g* for 10 min and, after removal of the broth, resuspended in PBS to a concentration of 2 x 10^6 CFU/ml, as calculated from the optical density at 600 nm. Subsequently, 50 µl of the bacterial suspension was mixed with 50 µl of PBS containing increasing peptide concentrations, from 0.13 µM to 16 µM, and 1% TSB. Next, the bacteria-peptide suspensions were incubated for 1 h, 2 h or 24 h at 37°C. To determine the number of viable bacteria, 10-fold serial dilutions were made and plated onto Diagnostic Sensitivity Test (DST) plates (Oxoid Ltd). Next IC_{99} values were calculated using linear regression analysis. For killing of biofilm-associated LUH14616, bacteria were mechanically removed from 24 h matured biofilms by scraping with pipet tips and vigorous vortexing. Subsequently, same procedure was followed as described above, (concentrations 0.5 - 32 µM) in PBS for 1 hr or 4 h.

Biofilm assay

Log-phase bacteria were diluted to 10^8 CFU/ml in biofilm adjusted medium, containing 62 mM potassium phosphate buffer (pH 7), 7 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 10 µM $FeSO_4$, 0.4% (wt/v) glucose, 0.5% (wt/v) casamino acids, further referred to as BM2 (36). Bacteria were cultured for 24 h in a flat-bottom polypropylene microtiter plate (Maxisorp, Greiner, Nurtingen, Germany) at 37°C, with or without antimicrobial peptides (concentrations 0.5 - 32 µM). Thereafter, biofilms were washed and stained using 1% (wt/v) crystal violet (Sigma-Aldrich). Crystal violet was eluted in 96% ethanol and the OD at 595 nm measured to quantitate the biofilm mass. Results are expressed as (EC_{50}).

Cell cultures

Cell cultures of normal human keratinocytes (NHK) and fibroblasts (NHF) were established from fresh human mamma or abdominal surplus skin, as described earlier (37, 38). In short, fat tissue was removed, and the skin was incubated with 2.4 U/ml dispase II (Roche, Woerden, The Netherlands), after which the dermis and epidermis were mechanically separated. NHK were isolated from the epidermis after treatment with 0.05% (wt/vol) trypsin (BD Falcon; Breda, The Netherlands) and cultured in Dulbecco's modified Eagle's medium (DMEM)

(Invitrogen, Breda, The Netherlands) diluted 3:1 in Ham's F-12 medium (Gibco, Bleiswijk, The Netherlands) supplemented with 5% (vol/vol) fetal calf serum (FCS) (HyClone Greiner), 100 U/ml penicillin-100 µg/ml streptomycin (Invitrogen), 1 µM hydrocortisone, 1 µM isoproterenol, and 0.1 µMinsulin (all Sigma-Aldrich), further referred to as keratinocyte medium. NHF were isolated after incubation in a 3:1 mixture of collagenase (Gibco) and dispase (Roche) for 2 h at 37°C and cultured in DMEM supplemented with 5% (vol/vol) FCS and penicillin-streptomycin, here called fibroblast medium.

Generation of human skin equivalents

Human skin equivalents (HSEs) were generated as described earlier (31). In short, 3 ml of rat-tail collagen (4 mg/ml) were mixed with 1.25×10^5 NHF. Next, the cells were pipetted onto a filter insert (6-wells, 0.4-µm filter; Corning, Amsterdam, The Netherlands) to allow polymerization of the collagen. The fibroblasts in collagen were cultured submerged in fibroblast medium for 2 days, after which 5×10^5 NHK/filter were seeded on top of the collagen layer. The cells were cultured under submerged conditions using keratinocyte medium supplemented with 1% (v/v) FCS, 1 µM selenious acid, 10 µM L-carnitine, 1 mM L-serine and a lipid mixture containing 25 µM palmitic acid, 15 µM linoleic acid, 7 µM arachidonic acid and 24 µM bovine serum albumin. After 2 days the HSEs were lifted to the air-liquid interface and cultured for an additional 10 days in serum free keratinocyte medium containing 30 µM linoleic acid and supplements as described as above. All medium supplements were purchased from Sigma-Aldrich. A representation of HSEs is presented in figure 1A and B.

Thermal wounding

HSEs were thermally wounded as described earlier (29,31). In brief, wounds were created on 10 days air-exposed models using a 2 by 10 mm blunt metal bar, which was placed in liquid nitrogen for 2 min. Immediately thereafter, the metal bar was applied onto the HSEs for 15 sec without any pressure, figure 1C demonstrates a periodic acid-Schiff (PAS)-alcian bluestaining of wounded HSEs.

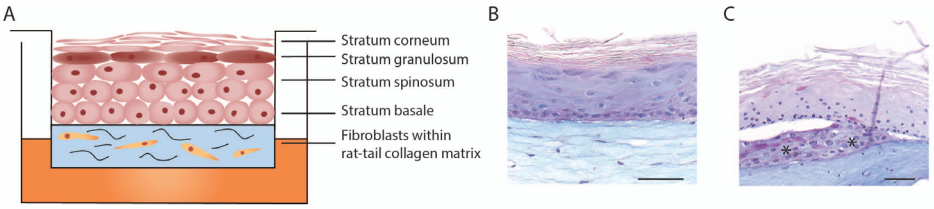

Figure 1. Schematic overview of HSE and thermal wounding of HSEs. (A) Schematic representation of HSEs showing the different layers of the epidermis and fibroblast-populated collagen layer on top of the filter insert. PAS-alcian blue staining of intact HSE (B) and HSE 24 h after wounding with liquid nitrogen (C). The asterisks indicate keratinocytes migrating over fibroblast-populated collagen layer, underneath the dead keratinocyte layer. Scale bars, 50 µm.

Wound infection and treatment

One hundred microliters of log-phase bacteria at a concentration of 1 x 10^6 CFU/ml in PBS were added to thermally wounded HSEs. After 1 h, the non-adherent and loosely adherent MRSA were removed by aspiration and the adherent bacteria were exposed to 100 µl peptide/antibiotic (1 mg/ml in PBS). At 4 and 24 h thereafter the numbers of viable detachable and adherent bacteria on the models were determined microbiologically using DST plates. The number of detachable bacteria was determined by washing one HSE, by pipetting 1 ml PBS on top of the model and pipetting this up and down 3 times. Next, serial dilutions of the peptides were made as described above, and plated onto DST plates. To assess the number of viable adherent bacteria, two punch biopsies (4 mm) were taken from each washed HSE, homogenized in 1 ml of PBS and serially diluted for CFU counting. The number of adherent bacteria per HSE (113 mm^2) was calculated by multiplying the number of adherent bacteria in two biopsies, 25.2 mm^2 (12.6 mm^2 per biopsy) by 4.5.

MTT assay

Epidermal skin equivalents, prepared as previously described (39), were exposed for 24 h to different peptide concentrations (50, 100, or 200 g of peptide/skin equivalent) at 37°C and 7.3% CO2. Next, the models were washed two times with PBS and then transferred to a fresh 12-well plate containing 600 µl of keratinocyte medium (prepared with transparent DMEM) containing 1 mg/ml 3-(4,5-dimethyl-2-thiazolyl)- 2,5-diphenyl-2H-tetrazolium bromide (MTT) (Sigma-Aldrich). After 3 h incubation at 37°C and 7% CO2, the MTT solution was removed and the models were washed twice with PBS. After air-drying for ≥2 h, 1 ml of isopropanol was pipetted on top of each insert and 1ml under each insert. Next, the 12-well plates were sealed with parafilm, stored overnight at

room temperature, and then shaken for ≥30 min on a plate shaker to extract all color. Thereafter, the extracts were transferred to a 96-well platefor measurement of the OD at 570 nm using a Tecan reader (Infinite F200; Männedorf, Germany). As a control 100 µl 100 mM SDS was applied onto the models.

Enzyme-linked immunosorbent assay

The protein level of IL-8 was measured (enzyme-linked immunosorbent assay [ELISA]) in the culture medium that was collected from HSEs (Invitrogen, Bleiswijk, The Netherlands). The ELISA procedures were carried out according to supplier's instructions. The lower limit of detection was 15 pg/ml.

Hematoxylin and eosin staining

Histochemical staining was performed on paraffin-embedded HSE sections. Slides (5 µm) were cut, deparaffinized, rehydrated, and washed with PBS. Subsequently sections were stained with hematoxylin (Klinipath, Duiven, The Netherlands) and eosin (Klinipath) and embedded in Depex (Serva biophoretics, Truckee, USA). Their morphology was assessed using a light microscope.

Statistical analysis

The data were analyzed by unpaired *t* test or for experiments using HSEs the Wilcoxon signed rank test. Differences between P values of ≤0.05 were considered significant. The IC_{99} values were calculated using linear regression analysis on the killing curves.

Results

Effect of LL-37, P60.4Ac and peptides derived thereof on log phase MRSA LUH14616

To assess whether substituting one or more amino acids in the sequence of P60.4Ac improves or decreases the antibacterial activity of this peptide, we compared the bactericidal activities of a set of 14 peptides derived from P60.4Ac toward MRSA LUH14616 in an *in vitro* killing assay. The peptides were incubated for 2 h with bacteria in PBS. The results revealed that P60.4Ac was more effective than LL-37 in killing LUH14616 in PBS (Table 1). Interestingly, only P10 killed >99% of the bacteria at a significantly (P = 0.02) lower concentration than did P60.4Ac, with a median (interquartile range) concentration of 1.1 (0.8 to 1.3) µM compared to 1.3 (1.1 to 1.4) µM, respectively. Based on these data, we selected P10 for further studies.

Table 1. Peptide sequences and *in vitro* killing of MRSA LUH14616 by a set of synthetic peptides in PBS.

Peptide	Sequence																								IC_{99} (μM)[a]
LL-37														L	L	G	D	F	F	R	K	S	K	E	K	
	I	G	K	E	F	K	R	I	V	Q	R	I	K	D	F	L	R	N	L	V	P	R	T	E	S	>16
P60.4Ac	I	G	K	E	F	K	R	I	V	E	R	I	K	R	F	L	R	E	L	V	R	P	L	R		1.3 (1.1-1.4)
P1	I	A	K	E	F	K	R	I	V	E	R	I	K	R	F	L	R	E	L	V	R	P	L	R		1.4 (1.2-1.5)
P2	L	A	R	D	Y	K	R	L	V	E	R	L	K	R	W	L	R	E	L	V	R	P	L	K		1.7 (1.5-1.8)
P3	I	A	K	E	F	K	R	I	L	E	R	I	K	R	F	I	R	E	I	T	R	P	I	R		2.1 (2.0-4.0)
P4	T	A	K	E	Y	K	R	I	L	D	R	I	K	R	Y	L	R	E	L	V	R	A	I	K		1.8 (1.8-2.2)
P5	V	A	K	D	Y	R	K	V	V	D	R	I	K	R	F	L	R	Y	L	L	R	P	V	R		1.2 (1.1-1.4)
P6	L	A	K	D	Y	K	K	I	V	E	R	L	R	K	W	L	R	E	V	L	R	P	V	K		1.9 (1.5-2.3)
P7	L	A	K	E	Y	R	K	I	F	D	R	L	K	K	W	L	R	Q	I	V	R	P	S	K		1.5 (1.0-1.6)
P8	L	A	K	E	W	R	K	I	V	D	R	L	K	R	W	L	R	D	I	L	K	A	T	K		1.2 (1.2-1.5)
P9	V	A	R	E	W	R	K	I	V	D	R	I	K	R	Y	L	R	D	I	S	K	A	T	K		4.3 (3.7-4.8)
P10	L	A	R	E	Y	K	K	I	V	E	K	L	K	R	W	L	R	Q	V	L	R	T	L	R		1.1* (0.8-1.3)[b]
P11	T	A	R	E	W	K	R	I	L	E	K	I	R	K	Y	L	R	D	V	S	R	V	T	R		5.4 (5.3-5.5)
P12	T	A	R	E	W	K	R	I	L	E	K	I	K	K	Y	L	R	D	V	S	R	V	T	R		5.1 (2.7-7.5)
P13	V	A	K	D	W	K	R	I	V	D	K	V	R	R	Y	L	R	E	V	T	K	I	L	K		3.2 (2.9-3.4)
P14	T	A	K	D	Y	R	K	I	F	E	K	I	K	K	Y	L	K	D	L	T	R	I	L	K		1.9 (1.6-3.2)

[a] Log-phase bacteria were exposed to increasing concentrations of the various peptides (range, 0 to 16 μM) for 1 h in PBS, after which the number of viable bacteria was determined microbiologically. The results are expressed as the 99% inhibitory concentration (IC_{99}), which is the lowest concentration of the peptide that results in a 99% reduction in the number of viable bacteria. IC_{99} values were calculated using linear regression analysis. The values are the median and range from >2 experiments, with the exception of P10 and P60.4Ac, which are from >9 experiments.

[b] Indicates value significantly different compared to that for P60.4Ac.

Effect of LL-37, P60.4Ac and P10 on different S. aureus strains

Next, we investigated the antibacterial activities of LL-37, P60.4Ac, and P10 toward four different *S. aureus* strains. For comparison, we included mupirocin, an antibiotic commonly used for topical eradication of (methicillin-resistant) *S. aureus* (40) in these experiments. We used both short (1-h) and long (24-h) incubation periods, and since *S. aureus* does not survive for 24 h in PBS, we supplemented the PBS with 1% TSB for these experiments. Already after 1 h, the synthetic peptides had killed >99% of the viable bacteria at concentrations ranging from 1.7 μM to 2.7 μM for P60.4Ac and 2.0 μM to 3.3 μM for P10 (Table 2). After 24 h, the IC_{99} values for the different strains ranged from 1.2 μM to 5.0 μM for P60.4Ac and 1.2 μM to 3.3 μM for P10 (Table 2). Interestingly, P60.4Ac and P10 were as effective as mupirocin against the different strains; moreover, P60.4Ac and P10 were alsowere also effective against the mupirocin-resistant strain LUH15051 having an IC_{99} concentration of 2.7 μM and 3.3 μM after 24 hrs incubation.

Effects of LL-37, P60.4Ac and P10 on biofilm formation

As biofilms can play a major role in infections, we further investigated the antibiofilm activities of these peptides. For this purpose, we first evaluated the abilities of LL-37, P60.4Ac, and P10 to inhibit the formation of biofilms on plastic. The results revealed that LUH14616 formed a large biofilm on plastic, while the other strains developed small/moderate biofilms (figure 2A). Therefore, we selected LUH14616 to test the effects of the peptides on biofilm formation. The results revealed a dose-dependent inhibition of biofilm formation by all three peptides; the maximum biofilm inhibition rates were 83% for LL-37, 85% for P10, and 86% P60.4Ac (figure 2B). P10 proved to be the most potent peptide for inhibiting biofilm formation, with a 50% reduction in biofilm formation (EC_{50}) at a median (interquartile range) of 2.0 μM (1.7 to 2.0 μM). This concentration was statistically significant (P = 0.002) compared to that of P60.4Ac, which had an EC_{50} of 2.8 μM (2.6 to 3.0 μM), and LL-37, which had an EC_{50} of 3.4 μM (2.6 to 3.7 μM). In addition, none of the peptides was bactericidal in the biofilm medium at the concentrations evaluated (data not shown), indicating that the inhibition of biofilm formation was not the result of killing the bacteria by the peptides.

Table 2. IC_{99} concentrations of LL-37, P60.4Ac, P10 and mupirocin on (methicillin resistant) *S. aureus* strains.

S. aureus strains	IC_{99} (μM) for peptides at[a]:							MIC (μg/ml)[b]
	1h			24 hrs				
	LL-37	P60.4Ac	P10	LL-37	P60.4Ac	P10	mup	
LUH14616	>16	2.0 (1.5-2.3)	2.0 (1.6-2.2)	>16	1.2 (1.1-1.3)	1.2 (0.3-1.4)	0.5 (0.4-0.6)	0.4
LUH15051	>16	2.7 (1.7-4.5)	2.9 (2.8-3.2)	>16	2.7 (1.7-4.5)	3.3 (0.8-5.3)	>64	>1024
Saco042	>16	2.4 (1.7-5.8)	3.3 (1.9-3.4)	>16	3.6 (2.6-5.4)	3.1 (2.3-6.2)	2.5 (0.4-7.7)	0.2
Imp-126	>16	2.2 (1.7-3.0)	2.0 (1.6-4.6)	>16	2.6 (2.0-6.5)	2.3 (1.1-2.7)	0.5 (0.4-0.5)	0.1
8325-4	>16	1.7 (0.7-2.2)	2.5 (0.8-3.3)	9.9 (7.7-10.1)	1.8 (1.7-2.0)	1.6 (1.1-2.2)	4.3 (3.6-4.8)	0.1

[a] Bacteria were incubated with peptides or mupirocin for 1 h or 24 h in PBS supplemented with 1% TSB. The results are expressed as the median (range) IC_{99} from 3 to 5 experiments. mup, mupirocin.

[b] MICs were assessed by Etest (bioMérieux).

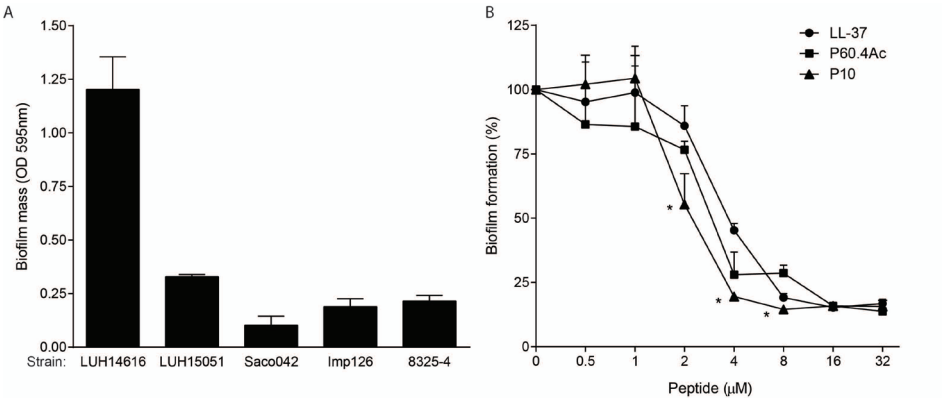

Figure 2. Biofilm formation by different *S. aureus* strains and inhibition of biofilm formation with LL-37, P60.4Ac and P10. (A) Biofilm formation by 5 *S. aureus* strains in BM2 medium for 24 h at 37°C was assessed using crystal violet staining. (B) Effect of peptides on biofilm formation by LUH14616 for 24 h. The results are shown as the percent biofilm formation compared to biofilm formation in the absence of peptides. The results are the medians and interquartile ranges of six independent experiments ($P < 0.05$). *, biofilm formation significantly different between P10 and the other peptides.

Effects of LL-37, P60.4Ac and P10 on established biofilm and biofilm-associated bacteria

Next, we tested the capability of the peptides to degrade established biofilms. For this purpose, we added peptides in PBS to 24-h-matured biofilms of MRSA LUH14616. After 4 h of exposure to the peptide, the number of viable bacteria was assessed. LL-37, P60.4Ac and P10 degraded biofilms in a concentration-dependent manner. Concentrations of >12.5 μM resulted in an approximately 25% reduction in the number of viable bacteria (figure 3A). As bacteria within biofilms are less sensitive to antibiotics than are log-phase bacteria, we determined the effects of the different peptides on biofilm-associated bacteria obtained by mechanical disruption of the biofilm. These biofilm-associated bacteria were treated with the peptides in PBS for 1 or 4 h, after which the number of viable bacteria was determined. Up to 32 μM, LL-37 did not reduce the number of viable biofilm-associated bacteria. Treatment for 1 h with P60.4Ac and P10, however, resulted in a >90% reduction in the number of viable bacteria at concentrations of ≥3.2 μM, while 4-h treatment resulted in a >2-log reduction in the number of viable bacteria with concentrations of ≥1.6 μM; moreover P10 was more effective than P60.4Ac (figure 3B and C).

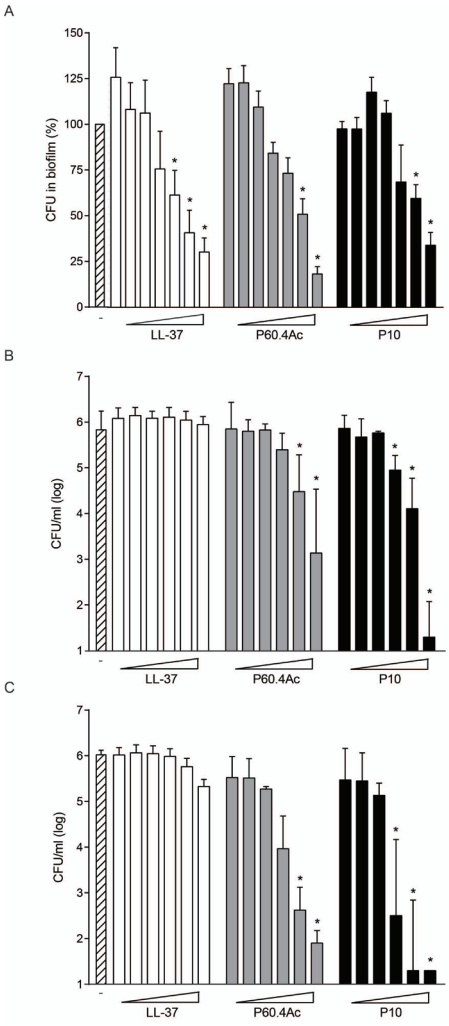

Figure 3. Effect of LL-37, P60.4Ac and P10 on biofilms and on biofilm-associated bacteria. (A) Breakdown of existing biofilms. Biofilms of LUH14616 were grown for 24 h and subsequently incubated for 4 h with peptides (concentrations, 1.5, 3, 6, 12, 25, 50, and 100 µM). Next, bacteria were scraped from the wells, and the number of viable bacteria was assessed microbiologically. The data are expressed as the percentage of surviving bacteria compared to the control biofilms. (B and C) Effects of peptides on biofilm-associated bacteria from 24-h-matured biofilms. The bacteria were scraped from the wells, diluted to 1 X 10^6 CFU/ml, and incubated for 1 h (B) and 4 h (C) with peptides (0.2, 0.4, 0.8, 1.6, 3.2, and 32 µM). White bars, LL-37; gray bars, P60.4Ac; black bars, P10; striped bar, control (no peptide present). The results are displayed as medians and interquartile ranges of 4 to 5 independent experiments. * indicates CFU/ml significantly less than that in the absence of peptide.

Antimicrobial peptides do not affect epidermal cell viability

To exclude the possible toxic effects of the peptides on HSEs, we determined the cell viability in the HSEs after exposure to increasing concentrations of the peptides by the MTT assay. Since the collagen layer of the HSE retains compounds like MTT, we used an epidermal skin model to assess the effect of the AMPs on mitochondrial activity. The application of LL-37, P60.4Ac, and P10 at concentrations up to 200 µg/model onto the stratum corneum for 24 h did not affect mitochondrial activity (figure 4A). Mupirocin also did not affect the epidermal viability (data not shown).

Next, we assessed the effect of the peptides on a thermally wounded HSE infection model (30). HSEs were thermally wounded using liquid nitrogen as shown in figure 1, and subsequently infected with 1 x 10^5 CFU/HSE. After topical application of 100 µg peptide in 100 µl PBS per thermally wounded and infected HSE, we observed no change in cell morphology, both in the unwounded part of the model (figure 4B). Moreover, we observed similar wound healing between treated models and untreated models, as observed by keratinocytes migrating over the wound bed (figure 4C, asterisks), underneath the dead keratinocyte layer (figure 4D, arrows).

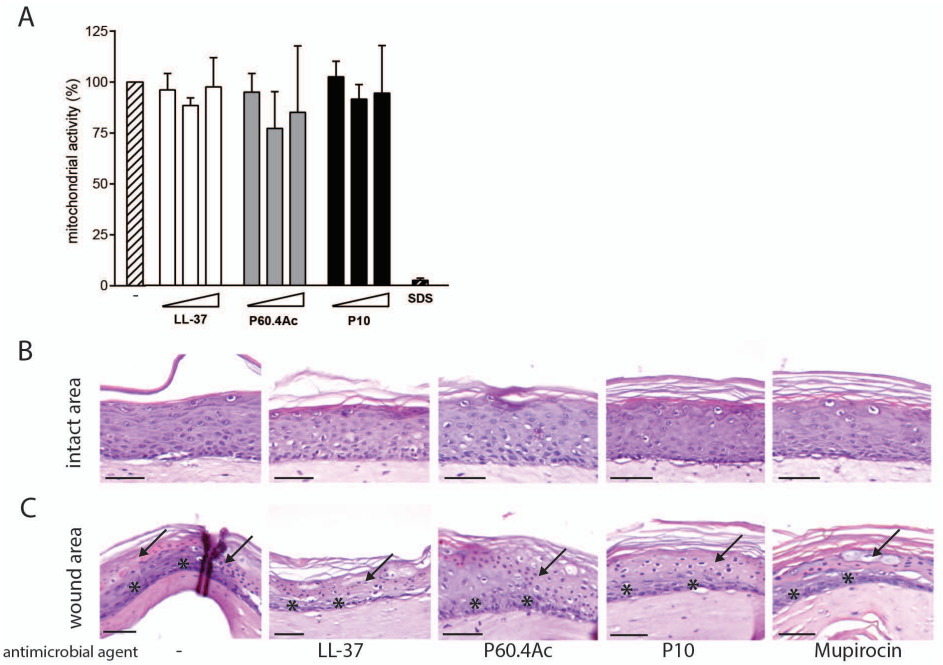

Figure 4. Effect of LL-37, P60.4Ac and P10 on mitochondrial activity and morphology. (A) MTT assay in epidermal skin models. Peptides LL-37, P60.4Ac, and P10 (50, 100, or 200 µg/model) or 100mM SDS was applied for 24 h onto the epidermal models. The results are shown as the percent cell viability compared to the untreated models and are presented as the medians and interquartile ranges of 4 independent experiments. White bars, LL-37; gray bars, P60.4Ac; black bars, P10; striped bar, control (no peptide present). (B to C) Hematoxylin and eosin staining of thermally wounded and LUH14616-infected HSEs exposed for 24 h to 100 µg/HSE of the various peptides and mupirocin, showing the intact area (B) and the wound area (C). The arrows mark the dead keratinocyte layer after thermal wounding, and the asterisks indicate keratinocytes migrating over the wound bed. Scale bars, 50 µm.

Effect of LL-37, P60.4Ac, and P10 on bacteria on thermally wounded HSEs

Based on these results, we concluded that the peptides are suitable to use for topical application. Next, we determined the effects of LL-37, P60.4Ac, and P10 on ther-

mally wounded HSEs infected with MRSA LUH14616. The infected HSEs were exposed to a single dose of 100 µg in 100 µl per HSE of LL-37, P60.4Ac, P10 or 100 µM (100 µl) mupirocin in PBS for 24 h. The preliminary experiments showed that 25 and 50 µg of the peptides per HSE were not effective (data not shown). Application of P60.4Ac, P10, and mupirocin, but not LL-37, were highly effective in reducing the number of viable LUH14616 on the wounded HSEs. While P60.4Ac reduced the CFU from 1.1 x 10^7 on untreated models to a median of 7.0 x 10^4 CFU/model (87% reduction), P10 reduced the number of viable bacteria to 2.3 x 10^3 CFU/model (>99.9% reduction); this difference was not significant.

To test whether the peptides were also effective against mupirocin resistant-MRSA, we performed the same experiments using mupirocin-resistant MRSA LUH15051. P60.4Ac and P10, but not LL-37 or mupirocin, were effective in eliminating LUH15051 for thermally wounded and infected HSEs, giving 96% and 99% reductions, respectively, in the number of viable bacteria (figure 5B). To determine the effects of the peptides on the inflammatory response induced by MRSA in the HSE, we measured the levels of excreted IL-8 in the supernatant. Exposure of the skin to MRSA LUH14616 resulted in an increased IL-8 production of 15.6 ng/HSE compared to 8.1 ng/HSE in the uninfected HSEs. Interestingly, we observed no effect of the various peptides on IL-8 secretion in HSEs (figure 5C), indicating that these peptides do not affect the inflammatory response in these models. Similar results were seen for mupirocin (data not shown).

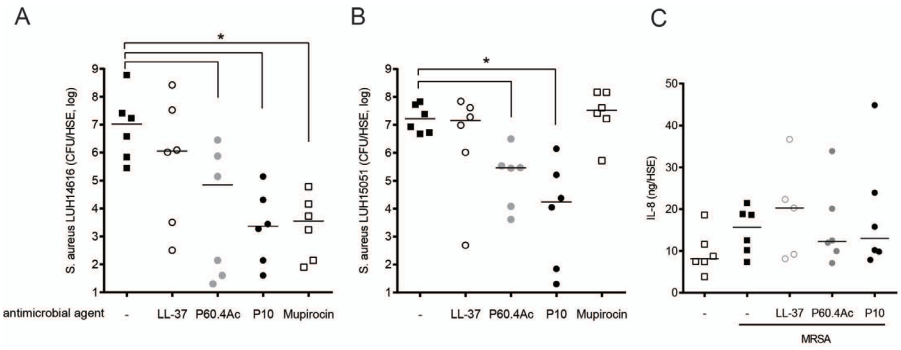

Figure 5. Effects of LL-37, P60.4Ac and P10 on MRSA and mupirocin-resistant MRSA infected thermally wounded HSEs. Thermally wounded HSEs were inoculated with LUH14616 (A) or LUH15051 (B) and after 1 h were subsequently exposed to 100 µg of the various peptides or mupirocin per model. After 24 h, the number of viable bacteria was determined microbiologically. (C) IL-8 in the culture supernatants of the HSEs wounded and infected with LUH14616 after 24 h of exposure to the peptides. ○, LL-37; gray circles, P60.4Ac; •, P10. Horizontal lines represent the median CFU counts. *, CFU/HSE significantly less than in colonized thermally wounded HSEs without antimicrobial agent (•).

Discussion

The aim of the present study was to develop new synthetic AMPs that may be used to reduce the bacterial burden of wounds, thus decreasing the risks of systemic infections and improve wound healing in, for example, burn patients (5). For this purpose we designed a set of 14 synthetic peptides with a predicted intact alpha-helical structure and net positive charge based on the amino acid sequence of the LL-37-derived peptide P60.4Ac (26). Our main finding is that only one of these peptides, i.e., P10, was more efficient than P60.4Ac in killing MRSA LUH14616. In agreement with this finding, we observed that P60.4Ac and P10, but not LL-37, were highly effective against five (methicillin resistant) *S. aureus* strains. Interestingly, P60.4Ac and P10 retained the antibiofilm activities of LL-37 (22). Moreover, P60.4Ac and P10 were more effective than LL-37 in eradicating MRSA and mupirocinresistant MRSA from infected wounded HSEs. Importantly, all three antibacterial peptides did not affect the viability of the keratinocytes in these models or trigger an inflammatory reaction. Based on these data, we conclude that P60.4Ac and P10 are interesting candidates for topical application to treat infected wounds.

LL-37 has many functions in the human immune defense systems, such as its roles in inflammation (41), wound healing (15, 25), LPS neutralization (24), and its antimicrobial activity against a variety of microorganisms (42, 43). Because of its immune-regulating and wound-healing properties, we aimed to develop synthetic LL-37-derived peptides that retain these beneficial properties while displaying enhanced bactericidal activities compared to that of LL-37. In line with this, it has been reported that the LL-37-derived peptide P60.4Ac was more effective against Gram-negative bacteria and fungi than LL-37 while retaining its LPS-neutralizing capabilities (26). In this study, we demonstrate that P60.4Ac and its derivative P10 are highly bactericidal toward (methicillin- resistant) *S. aureus*, an important pathogen in burn wound infections, whereas LL-37 is not. In addition, the two synthetic peptides were more effective than LL-37 against biofilmassociated bacteria derived from mechanically disrupted biofilms, reaching IC_{99} values after 4 h of incubation similar to the concentrations needed to kill log-phase bacteria. However, P60.4Ac and P10 were as effective as LL-37 in eradicating MRSA organisms residing within a 24-h biofilm. Of note, the effective concentrations of the various peptides when directly applied onto biofilms were much higher than those needed to kill planktonic bacteria, as has been extensively described for conventional antibiotics (11).

It has been established that LL-37 can prevent biofilm formation and attachment of bacteria to surfaces (20, 22). Other synthetic cationic peptides, named antimicrobial peptidomimetics, have been shown to degrade existing *S. aureus* biofilms (44). Here, we demonstrate that the synthetic peptides P10 and P60.4Ac can prevent biofilm formation as well as LL-37 can. All these results together suggest that synthetic peptides can degrade biofilms and subsequently eliminate bacteria in biofilms.

One of the aims of our study was to develop synthetic AMPs that can be used to reduce the bacterial burden of wounds. To investigate this, we used an *in vitro* infection model in which we inoculated thermally wounded HSEs with MRSA (45). Topical application of P60.4Ac and P10 significantly reduced the numbers of viable MRSA and mupirocin-resistant MRSA organisms from these surfaces, indicating that these peptides are suitable for topical application. However, it should be realized that these HSEs lack various characteristics of human skin, such as sweat glands, hair follicles, immune cells, and blood circulation. Because of these limitations, the results obtained in the described *in vitro* wound infection model may not fully reflect the effects of the peptides on (wounded) human skin. Nevertheless, we hypothesize that the results obtained using this model are more relevant for human wound infection treatment than those from testing the peptides in buffers or body fluids. However, we are aware that body fluids, such as plasma, may affect the effectiveness of the antimicrobial activities of the peptides, for example by binding to plasma components, such as albumin (46).

It has long been established that LL-37 can enhance wound healing (reviewed by Steinstraesser et al. [47]), and recently, a phase II study demonstrating enhanced wound healing in patients with chronic wounds was successfully completed (see above). In agreement with these findings, others have demonstrated that a bovine cathelicidin-derived peptide, IDR1018, can aid in wound healing in *S. aureus*-infected murine wounds (48). In addition, another LL-37-derived synthetic peptide, named IG-19, decreased disease severity and significantly reduced the serum levels of antibodies against collagen type II in a collagen-induced arthritis model (49). Besides allowing for an assessment of the antibacterial activities of peptides on infected tissues, the advantage of using an *in vitro* infection model that mimics the human skin is that the possible effects of the peptides on inflammation (as exemplified by IL-8 release), cell viability, and wound healing can be monitored in the same model. This is of particular importance when studying the effects of agents that affect various processes that are interlinked, such as the inflammatory reaction and wound healing. In line with this, we observed no effects of

P60.4Ac and P10 on IL-8 production by wounded HSEs in response to MRSA. Furthermore, no effects of these peptides on mitochondrial function or epidermal morphology in these models have been observed. It should also be noted that P60.4Ac has already been successfully applied to patients suffering from otitis media, and no negative side effects of this peptide were reported (27). Moreover, a dose up to 1.5 mg/kg of body weight/day of P10 has been administered intravenously to rats and miniature pigs without a negative impact on the animals (P. H. Nibbering, unpublished data).

Together, our results demonstrate the potential of the synthetic antibacterial peptides P60.4Ac and P10 as novel therapeutic agents for the treatment of wound infections. Moreover, these peptides may have additional beneficial effects, such as anti-inflammatory activities and enhancement of wound healing. This makes these synthetic antimicrobial peptides promising candidates, either alone or in combination with other antimicrobials (50), for the treatment of wound infections caused by (multidrugresistant) bacteria.

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Disclosure statement

The authors state no conflict of interest.

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