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Eradication of meticillin-resistant *Staphylococcus aureus* from human skin by the novel LL-37-derived peptide P10 in four pharmaceutical ointments

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ABSTRACT

Skin bacterial colonization/infection is a frequent cause of morbidity in patients with chronic wounds and allergic/inflammatory skin diseases. This study aimed to develop a novel approach to eradicate meticillin-resistant *Staphylococcus aureus* (MRSA) from human skin. To achieve this, the stability and antibacterial activity of the novel LL-37-derived peptide P10 in four ointments was compared. Results indicate that P10 is chemically stable and antibacterial in hypromellose gel and Softisan-containing cream, but not in Cetomacrogol cream (with or without Vaseline), at 4 °C for 16 months. Reduction in MRSA counts on Leiden human epidermal models (LEMs) by P10 in hypromellose gel was greater than that of the peptide in Cetomacrogol cream or phosphate buffered saline. P10 did not show adverse effects on LEMs irrespective of the ointment used, while Cetomacrogol with Vaseline and Softisan cream, but not hypromellose gel or Cetomacrogol cream, destroyed MRSA-colonized LEMs. Taking all this into account, P10 in hypromellose gel dose-dependently reduced MRSA colonizing the stratum corneum of the epidermis as well as biofilms of this bacterial strain on LEMs. Moreover, P10 dose-dependently reduced MRSA counts on ex-vivo human skin, with P10 in hypromellose gel being more effective than P10 in Cetomacrogol and Softisan creams. P10 in hypromellose gel is a strong candidate for eradication of MRSA from human skin.

1. Introduction

Acute bacterial skin and skin structure infections and bacterial colonization of chronic wounds and inflammatory skin diseases, such as atopic dermatitis, are major causes of morbidity. The most frequently encountered species in skin colonization and infection is (meticillin-resistant) *Staphylococcus aureus* (MRSA). The increase in antibiotic resistance requires the development of novel antimicrobial agents and/or strategies to prevent and combat skin and wound colonization and subsequent infection by such bacteria. Despite the recent introduction of several new antibiotics for the treatment of skin infections [1], there remains a need for novel antimicrobial agents. Due to their mode(s) of action being different from those of current antibiotics, antimicrobial peptides are considered as highly relevant candidates for the development of novel antibacterial agents [2–5]. As such, the authors have developed a highly effective synthetic antimicrobial peptide P60.4Ac based on the amino acid sequence of human cathelicidin LL-37 [6–8]. In an attempt to develop novel peptides with improved bactericidal efficacy, a panel of synthetic peptides which differ at a single position or at multiple positions from the sequence of P60.4Ac was designed and tested for bactericidal activity. Preliminary results revealed that peptide 10 (P10) was the most efficient peptide from this panel (Nibbering et al., unpublished data). The bactericidal activity of P10 and P60.4Ac exceeded that of LL-37 against all bacterial strains tested, while P10 was more effective than P60.4Ac in

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killing MRSA on wounded human skin models [7]. LL-37, as well as these two synthetic peptides, rapidly and effectively killed an array of bacteria irrespective of their resistance to current antibiotics, and displayed good in-vitro antibiofilm activities [6,7,9]. Based on these results, the authors intend to develop P10 for topical application (e.g. prevention and treatment of bacterial colonization and subsequent infection of wounds [10], and colonization of lesional sites on patients with atopic dermatitis [11–13]). For this purpose, the peptide needs to be incorporated into an easily applied, spreadable and stable ointment allowing for the prolonged and controlled release of the peptide at the target site. Since the stability of the peptide and the formulation are both critical for effective topical therapy, topical formulations – ranging from a simple gel and an oil-in-water cream to a water-in-oil cream – that have already been proven stable in clinical practice were considered. Therefore, this study focused on comparing: (1) the stability and antibacterial activity of P10 in four pharmaceutically, chemically and physically different ointments; and (2) the efficacy of the P10-containing ointments against bacteria and bacterial biofilms on human skin models.

2. Materials and methods

2.1. Synthesis and purification of P10

The N-terminal acetylated, C-terminal amidated peptide LAREYKKIVEKLKRYLRGVLRYLR (Mw 3137,89 Da), further referred to as peptide(P)10, was synthesized by 9H-fluorenylmethyloxycarbonyl chemistry as described previously [14]. Purification of the peptide was performed on a C18 column using a Prep4000 LC system equipped with a dual-wavelength ultraviolet (UV) detector and a FCIII fraction collector (all from Waters Chromatography BV, Etten-Leur, The Netherlands). Peptide-containing fractions were analysed by gradient reverse-phase ultra-performance liquid chromatography (UPLC) using an Acquity UPLC system equipped with a PDA detector and a SQD mass spectrometer (all from Waters). Data of the UV chromatograms at 220 nm in combination with the mass spectrometry spectra were used to select the purest fractions for combination in the final peptide pool solution. The peptide concentration in this solution was assessed using quantitative amino acid analysis (Eurosequence BV, Groningen, The Netherlands).

2.2. Compounding of the ointments

The peptide (375 mg) was dissolved in distilled water (3.75 mL) in a polypropylene conical tube (Greiner bio-one Cellstar tube; Greiner Bio-One BV, Alphen a/d Rijn, The Netherlands) to give a peptide stock solution with pH of 7.19. This solution was then worked up into four ointments. The gel base consisted of 3% w/v hypromellosum 4000 mPa.s and 30% v/v propyleneglycol in purified water. The Cetomacrogol cream base was comprised of 21.43% w/v cera cetomacrogolis emulsificans, 28.57% v/v cetiol V, 5.71% v/v the humectant sorbitolum liquidum cristallisable, 0.29% v/v sorbic acid and purified water (0.29%, v/v). The 20% v/v white Vaseline Cetomacrogol cream base consisted of 32.14% w/v vaselinum album, 21.42% v/v cera cetomacrogolis emulsificans, 17.86% v/v liquid paraffin, 0.29% v/v sorbic acic and 28.29% v/v purified water. The water-in-oil cream base was comprised of 14.28% v/v white Vaseline, 28.57% v/v liquid paraffin (Softisan 649) and 14.29% v/v purified water. All ingredients except Softisan 649 (IMCD Benelux, Rotterdam, The Netherlands) were from Duchefa Pharma (Haarlem, The Netherlands). The peptide solutions were mixed in these four bases to obtain ointments with the indicated dose of P10 and subsequently transferred to sterile, 5-g eye ointment tubes (Spruyt Hillen BV, Ijsselstein, The Netherlands). Softisan cream was transferred to a 20-mL polypropylene pot (Blocklandpack, Blockland BV, Maarsen, The Netherlands) because of the cream's physical instability. The placebo ointments consisted of the gel or cream base (70% w/v) and purified water (30% v/v). The four ointments containing 2% w/w P10 were stored at 4 °C for up to 16 months. Subsequently, various doses of P10 (range 0.03–1% w/w) in hypromellose gel were stored in the refrigerator (2–8 °C) and at room temperature (15–25 °C) for up to 19 months.

2.3. Analysis of the stability of P10 in the various ointments

The stability of P10 in the four ointments was determined using the isocratic high-performance liquid chromatography (HPLC) method developed for assessment of peptide P60.4Ac in Softisan cream [15] with minor modifications. In short, 50 mg of the hypromellose gel containing 2% w/w P10 was weighed in a 10-mL glass reagent tube. Next, 1 mL of internal standard [IS; 0.35 mg of spironolactone (Bufa, Spruyt-Hillen)/mL of acetonitrile:water:TFA=500:500:1] and 4 mL of purified water were added. This mixture was shaken till homogeneity and a 20-µL aliquot was injected into the HPLC apparatus. Separation of the sample components was performed on a Nucleosil C18 column, and isocratic elution was performed with water:acetonitrile:TFA=500:500:1 as eluent. The detection was performed at λ of 220 nm. The flow was 1 mL/min and the pressure was 138±4 bar. For analysis of P10 content of the two Cetomacrogol creams, 50 mg of the cream containing 2% w/w P10 was dissolved in 1 mL of methanol. After addition of 1 mL of spironolactone and 3 mL of purified water, the emerging emulsion was homogenized in an ultrasonic bath for 5 min, then shaken and a 2.5 mL-sample was centrifuged (10 min, 10,000 x g). After cooling, 20 µL of the water phase was injected into the HPLC apparatus. Finally, 50 mg of Softisan cream containing 2% w/w P10 was mixed with 2 mL of IS, i.e. sodium phenobarbital (0.03 mg/mL; Bufa) in purified water, and 0.5 mL of purified water. The mixture was placed in a 55°C water bath for 10 min and shaken three times for 30 s during this interval. Thereafter, samples were centrifuged (10 min, 10,000 x g), cooled and 20 µL of the water phase was injected into the HPLC apparatus. The peptide content of all four ointments was calculated from the standard curve (0.1–0.6 mg of P10/mL) prepared in distilled water.

2.4. Measurement of the release of P10 from gel

The release of P10 from hypromellose gel was determined by the disc assembly method using an Erweka light standard dissolution apparatus equipped with 200-mL dissolution vessels and a mini paddle per vessel. In all experiments, the paddle speed was 100 rpm. Approximately 1 g of gel containing 0.5% w/w P10 was introduced in a 1-mL Teflon chamber, which was covered with a polypropylene membrane filter (Sterlitech PP10047100, pore size 10 µm; Kent, WA, USA). The dissolution medium (water) was kept at 37±0.2 °C during the test. At various intervals, a 1-mL sample was taken for analysis, and subsequently, 1 mL of water was added to the dissolution vessel. The quantitative analysis was performed on a 200-µL sample using Jasco HPLC apparatus and ChromNav Chromatography Data System (Utrecht, The Netherlands). Separation of the components was performed on a Reprosil-Gold C18 column (Dr. Maisch, Ammerbuch, Germany) using a gradient of 95/5 A/B (t=0) to 10/90 A/B (t=7) (eluent A being Milli-Q with 0.1% v/v formic acid and eluent B being acetonitrile with 0.1% v/v formic acid). A standard curve using a range from 0.125 to 2.5 pmol P10/µL Milli-Q was used to quantify the P10 content of the samples.

2.5. Meticillin-resistant S. aureus

MRSA LUH14616 (sequence type 247) was maintained in nutrient broth (Thermo Fisher, Bleiswijk, The Netherlands) supplemented with 20% glycerol at -80°C. Inoculi from frozen stocks were grown overnight at 37°C on sheep blood agar plates (bioMérieux Benelux BV, Zaltbommel, The Netherlands). For experiments, mid-log phase bacteria were obtained by culturing the bacteria for 2.5 h at 37°C in tryptic soy broth (Thermo Fisher) under slow rotation.

2.6. In-vitro killing assay

The in-vitro killing assay [16] with minor modifications was used. In short, 180 µL of 2×10^5 colony-forming units (CFU) MRSA/mL of PBS supplemented with 1% v/v tryptic soy broth was added to polypropylene tubes (Micronic, Lelystad, The Netherlands) containing 20±1 mg of the various ointments with increasing amounts of peptide (0-0.1%, w/w). For comparison, 180 µL of the bacterial suspension was mixed with 20 µL of PBS containing similar amounts of P10 (0–32 µM). Next, the bacteria–peptide mixtures were incubated for 1 h or 24 h at 37°C, and thereafter the number of surviving bacteria was determined microbiologically using DST plates (Thermo Fisher).

2.7. Ethics statement

All primary human epidermal cells used in this study were isolated from surplus tissue collected according to Article 467 of the Dutch Law on Medical Treatment Agreement and the Code for Proper Use of Human Tissue of the Dutch Federation of Biomedical Scientific Societies. The Declaration of Helsinki principles were followed when working with human skin.

2.8. Exposure of MRSA-colonized Leiden human epidermal models to P10 in ointments

Leiden epidermal models (LEMs) (Biomimiq-Aeon Astron Europe BV, Leiden, The Netherlands) were inoculated on the air-exposed surface with 1×10^5 viable MRSA in 300 µL of PBS at 37°C in 7.3% CO_2. After 1 h, the bacterial suspension was aspirated to remove non-adherent bacteria. Next, approximately 20 mg of the different ointments containing various amounts of P10 or approximately 20 mg of placebo ointments – and, as controls, 100 µL of P10 (1 mg/mL of PBS) or 100 µL of PBS or 20 mg of mupirocin cream (2% w/w mupirocin in water in oil) – were applied on the colonized LEMs. After 1 h at 37 °C, the number of viable bacteria (CFU/mL) was assessed microbiologically. For this purpose, each LEM was homogenized in 1 mL of PBS using a glass Potter-Elvehjem tissue homogenizer, and the homogenates were serially diluted before transfer to DST plates. Where indicated, MRSA was allowed to form a biofilm on the LEMs for 24 h before application of the peptide-containing or placebo ointments. After 4 h at 37 °C, the number of viable bacteria residing within the biofilm was assessed as above.

2.9. Exposure of MRSA-colonized ex-vivo human skin to P10 in ointments

After removal of subcutaneous fat tissue, human donor skin was cut into small (2 × 2 cm) pieces. These pieces of skin were placed in filter inserts (Corning 3450; 0.4 µm; Corning Life Sciences, Amsterdam, The Netherlands); the stratum corneum being air-exposed at 37°C and the stratum basale in contact with culture medium. This medium consisted of Dulbecco's modified Eagle's medium/Ham's F12 (3:1) (Invitrogen, Thermo Scientific) with 5% v/v fetal calf serum (Hyclone, Thermo Fisher), 1 µM hydrocortisone and 1 µM isoproterenol (both Sigma-Aldrich, Zwijndrecht, The Netherlands), and penicillin/streptomycin (Invitrogen), and was refreshed twice per week. Approximately 24 h before the experiment, the medium was replaced by the same medium without antibiotics. Next, the skin models were inoculated with 1×10^5 CFU MRSA in 10 µL of PBS, and 20 mg of the various ointments was applied on the models after 1 h. At 1 h thereafter, the number of viable bacteria on the models was assessed as above.

2.10. Metabolic activity

LEMs were exposed for 24 h to the various P10-containing ointments – or as controls to mupirocin cream, no ointment or 100 µL of 100 mM SDS – at 37°C, then washed twice with PBS and transferred to a fresh 12-well plate containing 600 µL of keratinocyte medium (prepared with colourless DMEM) containing 1 mg MTT (Sigma-Aldrich)/mL. After 3 h at 37°C, the MTT solution was removed and the LEMs were washed twice with PBS. After air-drying, 1 mL of isopropanol was pipetted on top of each insert and 1 mL under each insert, the 12-well plates were sealed with parafilm, stored overnight at room temperature, and then shaken for at least 30 min on a plate shaker to extract all oxidized MTT. Thereafter, the optical density of these extracts at 570 nm was measured using a Tecan reader (Infinite F200, Thermo Fisher).

2.11. Histology and immunohistochemical staining for loricrin

LEMs were fixed in 3.7% formaldehyde and subsequently dehydrated and embedded in paraffin. Next, 5-µm tissue sections were cut and stained with haematoxylin/eosin (Klinipath BV, Duiven, The Netherlands). For immunohistochemical staining, 5-µm tissue sections were boiled in 10 mM citrate buffer, blocked with 2% v/v normal human serum (Sanquin, Leiden, The Netherlands) for 60 min. Next, the sections were adjusted to PBS and then incubated with 1000x diluted rabbit anti-loricrin antibody (AF62, Convance, Rotterdam, The Netherlands) overnight at 4°C. Thereafter, the sections were incubated with 200x diluted biotinylated swine anti-rabbit immunoglobulin (Dako Nederland BV, Heverlee, Belgium) for 1 h. After washes, the tissue sections were exposed to the streptavidin-biotin-peroxidase system (GE Healthcare). Finally, tissue sections were stained with 3-amino-9-ethylcarbazole, counterstained with haematoxylin, and sealed with Kaiser's glycerin.

3. Results

3.1. Stability and antibacterial activity of P10 in the four ointments

An ointment for slow release of P10 is a promising strategy to eradicate bacteria from human surfaces. As such, the peptide's stability and bactericidal activity were first assessed in four ointments upon storage for up to 16 months at 4 °C. HPLC analysis showed that P10 was chemically stable in hypromellose gel during this storage period (Table 1, Column 2). In addition, P10 at doses ranging from 0.03% to 1% remained stable in hypromellose gel at room temperature for at least 19 months (data not shown). In contrast, the peptide was chemically stable in Cetomacrogol cream for approximately 4 months at 4 °C. Thereafter, the cream became yellow which coincided with a gradual decrease in its P10 content, although no degradation products of P10 could be detected (Table 1, Column 3). In agreement, P10 was stable in Cetomacrogol cream with Vaseline for at least 2 months at 4 °C, and thereafter the peptide content dropped to 81% at Month 14 (Table 1, Column 4). The peptide was stable in Softisan cream for a period of at least 14 months at 4 °C (Table 1, Column 5), which was similar to that in the gel. Of note, after 4 months, evaporation of the water phase

Table 1
Stability of P10 in the four formulations upon storage.

Storage (months)	Peptide yields from ointments (%)			
	Hypromellose gel	Cetomacrogol cream	Cetomacrogol cream with Vaseline	Softisan 649 cream
0	100	103	95	104
1	101	98	95	111
2	102	nd	98	117
4	nd	86	nd	nd
7	102	nd	nd	nd
10	104	nd	nd	nd
14	99	nd	81	130
16	104	72	nd	nd

nd, not determined.
The peptide was isolated from the four ointments containing 2% w/w P10 at various intervals during storage at 4 °C. The stability of the peptide in the four ointments was assessed by high-performance liquid chrmatography analysis, and the peptide content was quantitated using a standard curve constructed with serial dilutions of the P10 stock solution stored at -20 °C. Results are means of two independent experiments.

Table 2
Bactericidal activity of P10 in four ointments.

Storage (months)	Exposure (h)	LC99.9 (μM)				
		Hypromellose gel	Cetomacrogol cream	Cetomacrogol cream with Vaseline	Softisan cream	PBS
0	1	1 (1–2)	32	32 (16–32)	8 (2–8)	2
0	24	1	16	32	12 (8–16)	2
16	1	2 (1–2)	32 (16–64)	32	8	2

The bactericidal activity of P10 in four ointments and, as control, phosphate buffered saline (PBS) was assessed using an in-vitro killing assay. Briefly, meticillin-resistant *Staphylococcus aureus* LUH14616 were mixed with the peptide in the various ointment bases/PBS and then incubated for 1 h and 24 h at 37 °C. Thereafter, the number of surviving bacteria was quantitated microbiologically. Results are expressed as the lethal concentration (LC) 99.9, i.e. the lowest concentration of the peptide that resulted in a 3-log reduction in the number of viable bacteria within 1 h and 24 h. Data are medians and ranges of three to five experiments.

as well as separation of the water phase and the fat phase was observed, indicating that this water-in-oil cream is physically instable. Studies into the ability of P10 in the four ointments to kill MRSA revealed that the bactericidal activity of P10 in hypromellose gel and PBS was comparable, whereas the peptide's bactericidal activity in the three creams was considerably reduced (Table 2). After 16 months of storage, the antibacterial activity of P10 in the four ointments was similar to the peptide's activity before storage (Table 2). Together, the stability and efficacy of P10 in hypromellose gel is better than in the three creams.

3.2. Effects of peptide-containing and placebo ointments on cell viability, morphology, cell differentiation and inflammatory responses in Leiden epidermal models

To determine if the various P10-containing and placebo ointments exert adverse effects on the epidermis, the effects of these ointments on the viability, morphology, differentiation and inflammatory responses of the cells in LEMs were assessed. Histological and histochemical staining revealed no effects of the various ointments on cell viability (Fig. 1A), epidermal morphology (Fig. 1B) and terminal differentiation (Fig. 1C). In agreement, the various ointments, irrespective of the presence of P10, did not trigger the expression of mRNA for IL-1α, IL-8, hBD-2 and hBD-3 by LEMs, as assessed by quantitative polymerase chain reaction, and IL-8 by enzyme-linked immunosorbent assay (data not shown). Next, potential adverse effects of the various (P10-containing) ointments on MRSA-colonized LEMs were investigated. For this purpose, LEMs were exposed to MRSA for 1 h before application of the four P10-containing and placebo ointments. Results revealed that irrespective of the presence of P10, the two fatty creams, but not hypromellose gel and Cetomacrogol cream, caused substantial thinning of the epidermal layers within 1 h (Fig. 1B) with poor expression of the terminal differentiation marker (Fig. 1C) and inflammation markers (data not shown). In line with these data, 2% w/w mupirocin in an emulsion (Bactroban®) was found to induce substantial thinning of the epidermal layer (data not shown). Exposure of the models to MRSA for 1 h before application of (P10-containing) hypromellose gel and Cetomacrogol cream revealed that the two P10-containing ointments, but not the placebo ointments, reduced MRSA counts effectively on the LEMs (Fig. 1D). Of note, P10-containing gel, but not P10 in Cetomacrogol cream, completely eradicated the bacteria from the LEMs (Fig. 1D).

3.3. Antibacterial effects of hypromellose gel containing P10 on MRSA on LEMs

As P10 in hypromellose gel is highly effective and does not exert adverse effects on MRSA-colonized LEMs, the effect of increasing doses of P10 (0.1–2% w/w) in gel on MRSA-colonizing LEMs was determined. Results revealed a dose-dependent reduction in bacterial counts on the LEMs, with complete eradication observed for gel containing ≥1% w/w P10 (Fig. 2A). In addition, gel containing 1% and 2% w/w P10, but not lower doses, degraded MRSA biofilms on LEMs significantly (Fig. 2B).

3.4. Release of P10 from hypromellose gel

Evaluation of the release of P10 from hypromellose gel by routinely used methodology [17] revealed rapid release during the first 2 h, followed by a levelling off to a maximum of >95% of the peptide released at 5 h (Fig. 3).

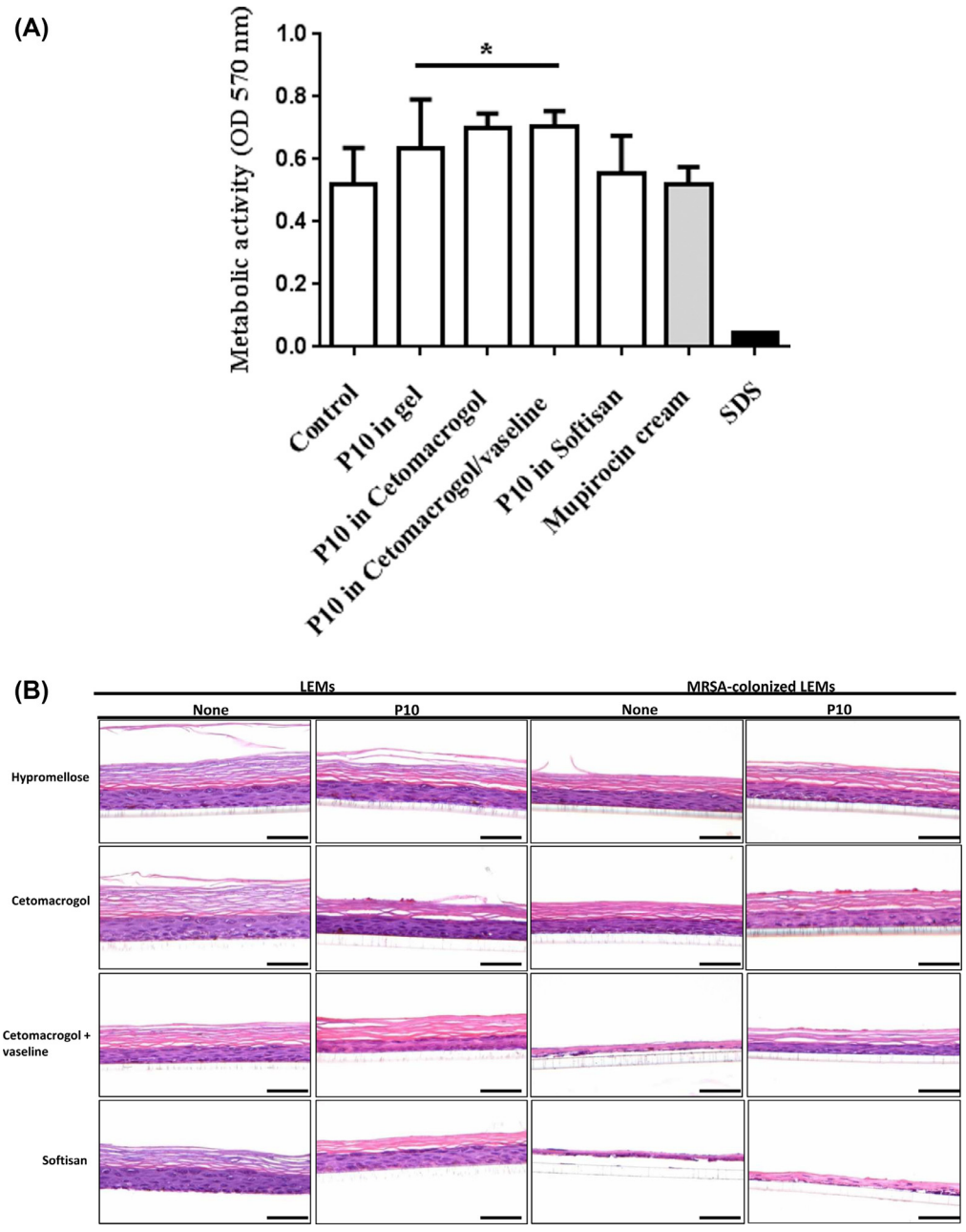

Fig. 1. Effects of P10-containing and placebo ointments on epidermal cell viability (A), epidermal morphology (B) and cell differentiation (C) in Leiden epidermal models (LEMs) and on the bacterial counts for meticillin-resistant *Staphylococcus aureus* (MRSA) on these models (D). (A) LEMs were exposed for 24 h to 20 mg of the various ointments containing P10 (2% w/w), and as controls to phosphate buffered saline (PBS), mupirocin-containing cream and SDS, and then the metabolic activity of the cells in epidermal models was assessed as an indication of viability. Briefly, after exposure to the ointments, the models were transferred to a fresh 12-well plate containing colourless medium with 1 mg/mL MTT. Three hours thereafter, the medium with surplus MTT was removed and the oxidized MTT was extracted from the models. Finally, the optical density (OD) of these extracts at 570 nm was measured. As positive control, 100 µL of 100 mM SDS was applied. Results for three different donors (each in triplicate) are displayed as means and standard errors of the means. $^{*}P<0.05$ significantly different from control (PBS). (B) LEMs were inoculated or not with MRSA LUH14616 and then exposed for 24 h to placebo or P10-containing (2% w/w) ointments. Thereafter, tissue sections of the epidermal models were cut and stained for haematoxylin-eosin. Results are of one representative donor out of three different donors. Bar=50 µm. (C) LEMs were inoculated or not with MRSA LUH14616 and then exposed for 24 h to placebo or P10-containing (2% w/w) ointments. Thereafter, tissue sections of the epidermal models were cut and stained for the terminal differentiation marker loricrin by immunohistochemistry. Results are of one representative donor out of three different donors. Bar=50 µm. (D) LEMs were exposed to 1×10^5 viable MRSA LUH14616 in 100 µL of PBS for 1 h. Thereafter, the non-adherent bacteria were removed by aspiration and the models with adherent bacteria were exposed to approximately 20 mg of hypromellose gel or Cetomacrogol cream containing 2% w/w P10 or the placebo ointments. In addition, colonized epidermal models were exposed to 100 µL of a peptide solution (1 mg/mL) or to PBS without peptide for 1 h. Finally, the epidermal models were homogenized and viable bacterial counts in these homogenates were determined microbiologically. Data are medians and interquartile ranges of three to seven experiments. Two-tailed Mann–Whitney U-tests indicated that the values for P10-containing ointment differed significantly from control (PBS) or placebo with $^{*}P<0.05$ and $^{**}P<0.01$. CFU, colony-forming units.

3.5. Antibacterial effects of P10 in hypromellose gel and Cetomacrogol and Softisan creams on MRSA colonizing ex-vivo human skin

Lastly, the effect of increasing doses of P10 (0.1–2% w/w) in gel on MRSA colonizing ex-vivo human skin was determined. As Cetomacrogol and Softisan creams do not exert adverse effects on human skin, the activities of increasing doses of P10 in these creams on MRSA on ex-vivo skin were also determined. Results indicated that P10 in all three ointments dose-dependently reduced the bacterial counts on the skin, with the peptide in gel

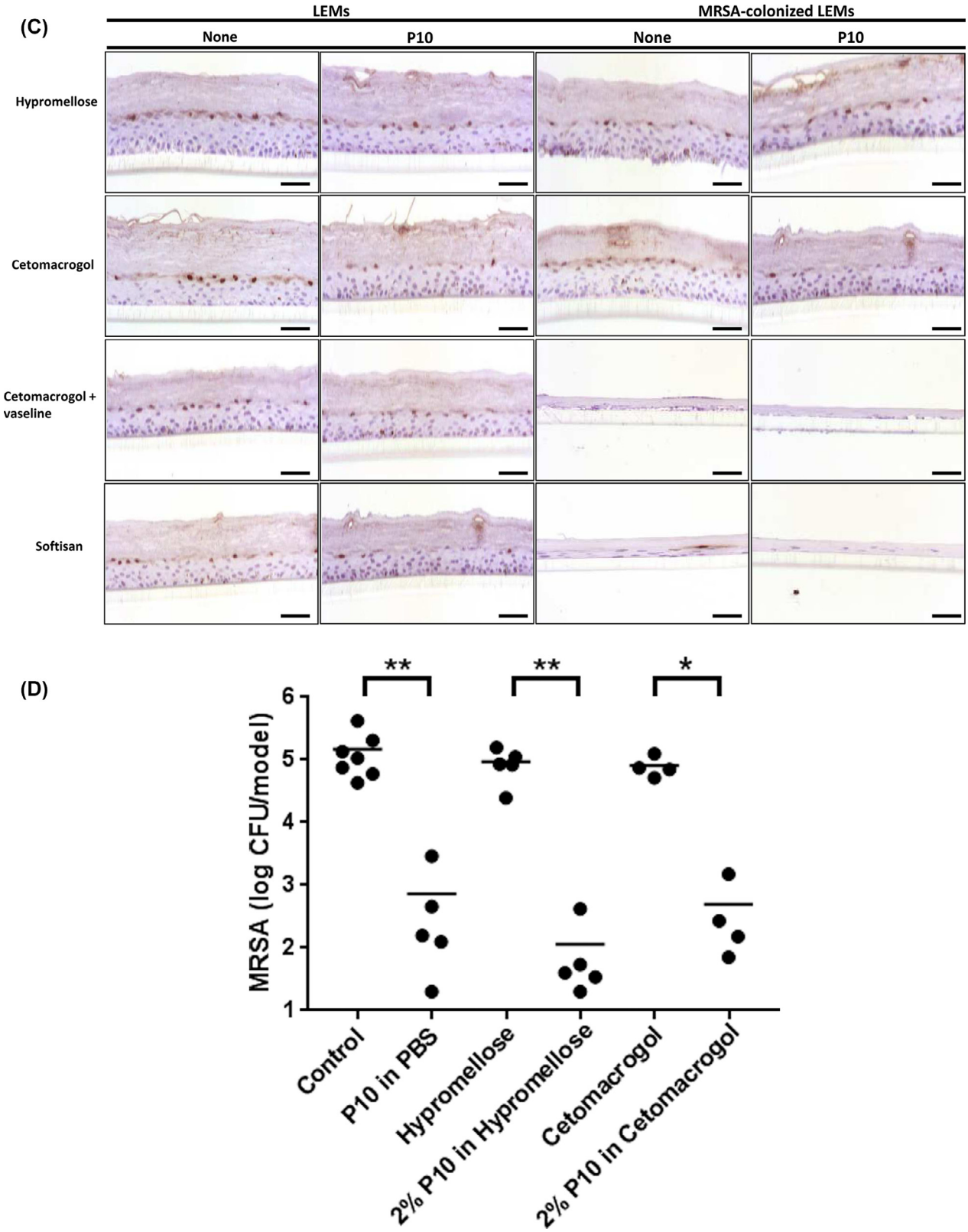

Fig. 1. Continued

being slightly more effective than in the two creams (Fig. 4). The maximal bactericidal effect of P10-containing gel was comparable with that of the standard care of treatment (i.e. mupirocin cream) (Fig. 4).

4. Discussion

The design of an ointment for slow release of the novel antimicrobial peptide P10 at the colonized/infected site is a

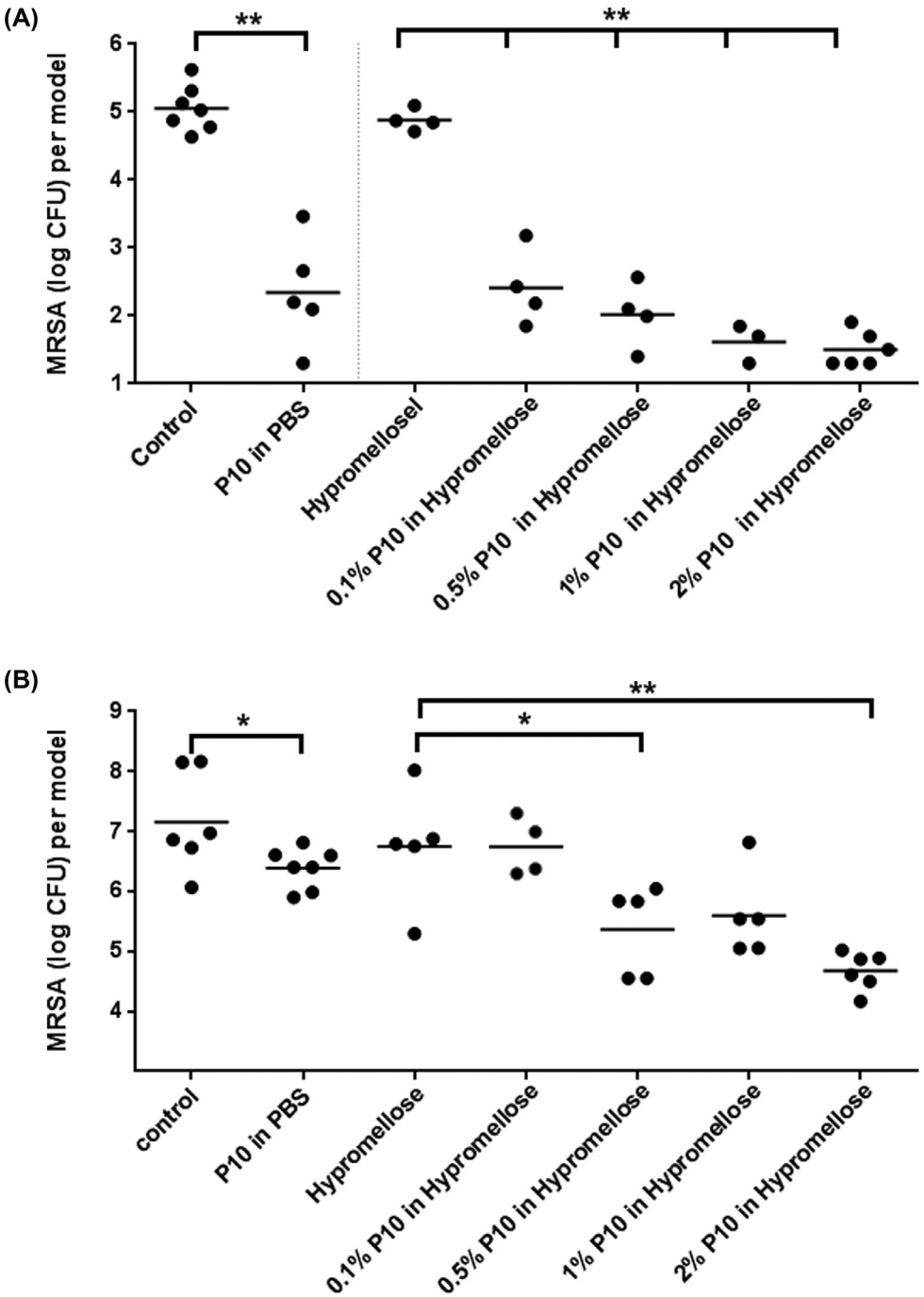

Fig. 2. Dose-dependent elimination of meticillin-resistant *Staphylococcus aureus* (MRSA) from Leiden epidermal models by P10 in hypromellose gel. (A) Effect of exposure of MRSA on epidermal models to increasing doses of P10 in hypromellose gel or as positive control P10 in phosphate buffered saline (PBS) (1 mg/mL) and negative control PBS for 1 h was quantitated microbiologically. Medians of the results for four different donors are indicated by the lines. Two-tailed Mann–Whitney U-tests indicated that the values for P10-containing ointment differed $^{**}P<0.01$ significantly from control (PBS) or placebo gel. (B) Effect of exposure of MRSA biofilms on epidermal models to increasing doses of P10 in hypromellose gel or as control PBS for 4 h was assessed microbiologically. Medians of the results for three to five different donors are indicated by the lines. Two-tailed Mann–Whitney U-tests indicated that the values for P10-containing ointment differed $^{*}P<0.05$ or $^{**}P<0.01$ significantly from control (PBS) or placebo gel. CFU, colony-forming units.

promising approach to eradicate (antibiotic-resistant) bacteria from human skin. This study worked up the peptide into four pharmaceutically, physically and chemically, different ointment bases that have already been developed successfully for topical application of other drugs to humans. The conclusion that hypromellose gel is the preferred ointment for P10 is based on the following findings. First, P10 in hypromellose gel was more effective on MRSA LUH14616 *in vitro* and such bacteria on ex-vivo human skin than this peptide in the three creams. Secondly, P10 is released from the gel rapidly and efficiently; within 2 h, more than 80% of the peptide was released, which was superior to the release from the three creams (unpublished data). Thirdly, gel containing P10 did not affect cell viability, inflammatory activities, morphology and differentiation of cells within LEMs, indicating that this peptide–gel combination has no adverse effects on the epidermis. Fourthly, P10 was found to be stable and effective in hypromellose gel for

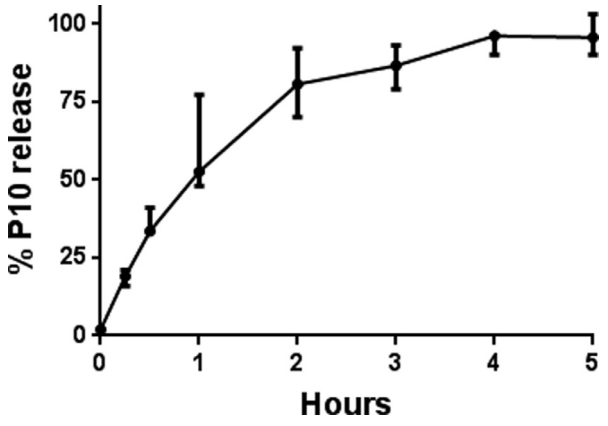

Fig. 3. Release of P10 from hypromellose gel was assessed by the disc assembly method using an Erweka light standard dissolution apparatus equipped with 200-mL dissolution vessels and a mini paddle per vessel. In all experiments, the paddle speed was 100 rpm. In short, 1 g of gel containing 0.5% w/w P10 was introduced in a 1-mL Teflon chamber, which was covered with a polypropylene membrane filter (pore size 10 µm). The dissolution medium (water) was kept at 37±0.2 °C during the test. After various time intervals, a 1-mL sample was taken from the dissolution vessel for analysis, and subsequently, 1 mL of water was added to the dissolution vessel. The quantitative analysis of the sample was performed by analysing 200 µL of the sample on an high-performance liquid chromatography apparatus. Results are medians (and ranges) of the values from two separate experiments (each in duplicate).

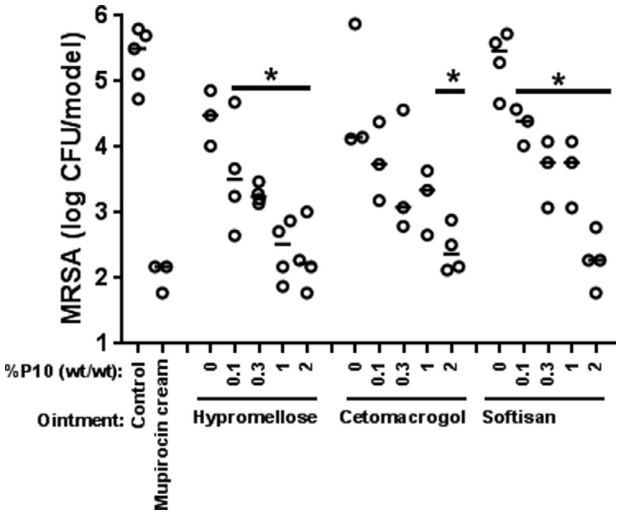

Fig. 4. After exposure of pieces of human skin to 1×10^5 viable meticillin-resistant *Staphylococcus aureus* (MRSA) LUH14616 in 10 µL of phosphate buffered saline for 1 h, the models with bacteria were treated with 20 mg of the various ointments with increasing doses of P10 or to the placebo ointments for 1 h. Thereafter, the models were homogenized and viable bacterial counts in these homogenates were determined microbiologically. Data are medians and interquartile ranges of three to five experiments. Two-tailed Mann–Whitney U-tests indicated that the values for P10-containing ointment differed $^*P<0.05$ significantly from placebo. CFU, colony-forming units.

at least 19 months at 4 °C and room temperature, which was not the case for the three creams. Not surprisingly, (hypromellose) gel has been introduced successfully as a topical delivery system for other antimicrobial peptides, such as LL-37 [18] and SAAP-148 [19]. Possible explanations for the main finding of this study (i.e. P10 in hypromellose gel eradicated MRSA from LEMs and ex-vivo human skin more efficiently than when formulated in Cetomacrogol and Softisan creams) may include enhanced killing of the bacteria by the peptide in the gel and/or optimal delivery of P10 at the site of infection. Indeed, this study found that P10 in hypromellose gel was more effective at killing bacteria *in vitro* than when formulated in Cetomacrogol and Softisan creams, and that the peptide was released in higher amounts from gel than creams. P10 in gel dose-dependently reduced the number of MRSA on LEMs and ex-vivo skin, with complete eradication seen after application of ≥1% w/w P10. In agreement, P10 in hypromellose gel was effective in eradicating MRSA from tape-stripped mouse skin *in vivo* (unpublished results). Since biofilms play an important role in infections [20] while displaying reduced susceptibility to most antibiotics [21] and immune effector mechanisms, the observation that P10 in gel dose-dependently reduced the biofilm mass on human skin models, with maximal reduction (>99%) seen with 2% w/w P10, is of clinical significance. However, it should be noted that P10 in hypromellose gel did not completely eradicate the MRSA biofilm from LEMs. The possibility that hypromellose gel can deliver a biofilm-dispersing agent (e.g. the antibiotic ADEP4 [22], lysostaphin [23] or maggot secretions [24]) together with P10 may be sought. Although the various ointment bases are already in (clinical) use, it was noted that the two fatty creams destroyed the MRSA-colonized LEMs but not the non-colonized models. It is possible that virulence factors produced by MRSA [25], such as α-toxin and lipases, render the models susceptible to the detrimental actions of these fatty creams.

Another important finding pertains to the chemical stability and bactericidal activity of P10 in the two oil-in-water creams. After a couple of months, these creams changed from white to yellow, and HPLC analysis showed a gradual decrease in P10 content corresponding with the occurrence of this colour change. No degradation products of P10 were found in the Cetomacrogol creams, which was confirmed by mass spectrometry. A possible explanation could be that P10 is trapped in the modified Cetomacrogol fraction. In agreement, repeated extraction of peptide from the yellow Cetomacrogol cream was found to result in increased peptide recovery.

Although no detrimental effects of the various ointments (with or without P10) on the viability of cells in LEMs were detected, the presence of the peptide in hypromellose gel and the two Cetomacrogol creams was associated with a significant increase in metabolic activity. Unfortunately, a reasonable explanation for this unexpected effect of P10 is not available at present.

As the models used in this study suffer from several limitations, including lack of immune cells, blood circulation and microbiome, the present results may not fully reflect the effects of P10 on patients with wound infections. In addition, it cannot be excluded that the activity of P10 on mature biofilms is less potent than portrayed here (based on the present results for immature biofilms). Nevertheless, the combination of the clinically used hypromellose gel and P10 is a good candidate for further development as a new approach to prevent bacterial colonization of body surfaces, and to treat topical infections caused by bacteria that do not respond effectively to current antibiotics.

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Ethical approval: All primary human epidermal cells used in this study were isolated from surplus tissue collected according to Article 467 of the Dutch Law on Medical Treatment Agreement.

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