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The functional stability, bioactivity and safety profile of synthetic antimicrobial peptide SAAP-148

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The synthetic antimicrobial peptide SAAP-148 was found to be effective against a wide range of (antimicrobial-resistant) gram-positive and gram-negative bacteria, *in vitro* and in a superficial skin infection model in mice, but not a surgical wound infection model in rats. In addition, the efficacy of SAAP-148 in protein-rich environments such as blood plasma and eschar extract is considerably reduced as compared to PBS. The aim of the present study is to determine which wound-related factors reduce SAAP-148's efficacy. As high dosages of SAAP-148 may be required in infected wounds we also assessed the *in vitro* and *ex vivo* cytotoxicity of SAAP-148. Preincubation of SAAP-148 in 50% (v/v) blood plasma or eschar extract for 24 h, 3% (v/v) bovine serum albumin or a collagen-elastin 3D matrix significantly reduced the bactericidal efficacy of the peptide. Also, the presence of protease inhibitors did not restore the bioactivity of SAAP-148, suggesting a reduced bioavailability of SAAP-148 due to protein binding, rather than degradation. Furthermore, relatively low concentrations of SAAP-148 (≥0.23 nmol/100 µl) induced cytotoxicity for human skin cells in 2D culture. Contrarily, SAAP-148 did not induce cytotoxicity for cells in *ex vivo* human skin. In addition, unlike silver sulfadiazine (SSD), SAAP-148 showed no statistically significant adverse effects on the re-epithelialization of *ex vivo* excision wounds and burn wounds. Hence, SAAP-148 potentially has a beneficial profile for antimicrobial treatment of infected wounds.

Key words: SAAP-148, proteolytic degradation, bioactivity, peptide interaction, cytotoxicity, skin and soft tissue infection.

INTRODUCTION

Bacterial colonization of burn wounds is common because the skin, which normally acts as a physical barrier, is compromised by the injury (Glik et al., 2012). Opportunistic bacteria such as *Staphylococcus aureus*

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have the ability to evade the immune responses (Rooijakkers et al., 2005), which can lead to uncontrolled bacterial colonization in burns. To prevent and control bacterial colonization in burn wounds, topical treatments such as silver sulfadiazine (SSD), mupirocin and fusidic acid in ointments have been developed (Atiyeh et al., 2007; Gould and Villiger, 1986; Williamson et al., 2017). However, limited bactericidal efficacy, emergence of drug-resistance or cytotoxicity for the host cells induced by these topicals has increased the need for novel antimicrobial agents.

In the fight against antibiotic-resistant bacteria, antimicrobial peptides (AMPs) have been identified as promising therapeutic agents (Gordon et al., 2005). AMPs are usually small cationic molecules ranging from 12 to 50 amino acids with an amphiphilic tertiary structure. They are effective against a wide range of bacteria as their amphiphilic characteristics allow them to penetrate into the bacterial membrane (Beisswenger and Bals, 2005; Shai, 2002). Due to this anticipated mechanism of action, resistance against AMP is less likely to occur (Zasloff, 2002). The variety in antibacterial efficacy that has been observed among different naturally occurring AMPs is related to the broad diversity in the amino acid sequence and secondary structure (Kumar et al., 2018). These structural properties of AMPs also determine their stability in biological environments and potential toxicity for human cells (Yin et al., 2012).

Previously, a novel synthetic AMP, known as SAAP-148, was identified as a promising agent to combat (resistant) bacteria (de Breij et al., 2018). This peptide effectively eradicated methicillin resistant *S. aureus* (MRSA) in a skin infection mouse model (de Breij et al., 2018). However, SAAP-148 did not eradicate MRSA in a partial thickness wound model in rats (Dijksteel et al., 2019a). We hypothesize that this effect was mainly caused by the protein and protease-rich wound environment of the open wound. Hence, the aim of the present study is to determine the functional stability and bioactivity of SAAP-148 in conditions mimicking the wound environment. As relatively high SAAP-148 concentrations are needed to eliminate bacteria in biological relevant environments, we also determined the cytotoxicity of such SAAP-148 concentrations for human cells in 2D culture and in *ex vivo* human skin, and estimated the therapeutic index.

MATERIALS AND METHODS

Ethylenediaminetetraacetic acid (EDTA) was purchased from Gibco-BRL (Life Technologies, N.Y. USA) and protease inhibitor mini tablets (EDTA-free) were purchased from Pierce Biotechnology (Rockford, UK). Novomaix™ is a collagen-elastin 3D scaffold, which was a kind gift from Matricel GmbH (Herzogenrath, Germany). SAAP-148 was obtained from the Leiden University Medical Center (Leiden, the Netherlands). For the synthesis, purification and identification of SAAP-148 see Nell et al. (2006). Lyophilized peptide was dissolved in phosphate-buffered saline (PBS; Gibco,

Paisley, UK) and stored in aliquots at −20°C until use.

Human blood plasma and tissue

Human blood and tissues were obtained according to institutional guidelines and medical research "code of conduct for responsible use", drafted by Federa (Foundation Federation of Dutch Medical Scientific Societies). Human blood plasma from three donors was obtained from Sanquin (Leiden, the Netherlands). The plasma was pooled, centrifuged at $2266 \times g$ for 10 min, filtered using 0.2 µm filters to remove aggregates and stored in aliquots of 50 ml at -20°C. Eschar or burn wound tissues was obtained from the Burn Center, Red Cross Hospital (Beverwijk, the Netherlands). An extract was prepared from eschar as previously described (Dijksteel et al., 2019a).

Prior to the experiment, blood plasma or eschar extract were thawed and centrifuged at $2738 \times g$ for 10 min. Proteins from blood plasma or eschar extract were precipitated using methanol at a final concentration of 90%. The protein suspension was centrifuged at $10000 \times g$ for 10 min and the pellet was washed and dissolved in PBS.

Cell cultures

The epidermis of 0.3 mm split-thickness skin grafts was mechanically separated from the dermis after 30 min incubation with 0.25% (wt/v) dispase (Gibco) in PBS at 37°C and 5% CO_2 using two forceps. Keratinocytes were isolated from the epidermis after 15-25 min incubation with 0.05% (wt/v) trypsin in 0.53 mM EDTA (Gibco), as previously described (Coolen et al., 2007). The dermis was cut into small pieces using a scissor. Fibroblasts were isolated from the dermal tissue after 2 h incubation with a 1:1 mixture of 0.25% (wt/v) collagenase and dispase, as previously described (Van den Bogaerdt et al., 2002). Keratinocytes were cultured in defined keratinocyte serum free medium (Gibco) supplemented with 1% (w/v) penicillin/streptomycin (P/S; Gibco); further referred to as keratinocyte medium. Fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS; Gibco), 1% (v/v) P/S and 1% Glutamax (Gibco); further referred to as fibroblast medium. Cells were seeded (12000 cells/ cm^2) onto tissue culture plastic (fibroblasts) or tissue culture plastic coated with 1 µg/cm^2 collagen type IV ((Merck KGaA) (keratinocytes) (Coolen et al., 2007). Cells were cultured at 37 °C and 5% CO_2 until 80-90% confluency was reached.

Ex vivo human skin models

Human skin was obtained from patients undergoing elective abdominal dermo-lipectomy. Skin grafts with a thickness of 0.8 mm were prepared using a dermatome (Aesculap AG & Co. KG, Tuttlingen, Germany). Grafts were stored in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco) supplemented with 2% (v/v) P/S (Gibco) at 4°C for a maximum of two weeks. Prior to the experiment, the grafts were washed in RPMI 1640 medium at 4°C overnight, followed by PBS for approximately 2 h at room temperature to ensure the removal of P/S.

Ex vivo excision wound models (EWMs) were prepared from skin grafts using a dermatome (width 7 mm) to remove 0.3 mm of the upper part of the skin graft containing the epidermis. Subsequently, the grafts were cut into pieces of 1 × 1.5 cm using a scalpel (Dijksteel et al., 2019a). To prepare burn wound models (BWMs) the skin graft was cut into pieces of 1 × 1 cm using a scalpel and a burn was inflicted on the skin piece by placing a soldering iron

(10 × 2 mm) set at 95°C on the epidermis for 10 s without exerting pressure.

Bacterial culture

A clinical isolate of MRSA (LUH14616; Haisma et al., 2014) was cultured in Luria Bertani (LB) medium at 37 °C and shaken at 200 rpm for approximately 4 h. The bacterial culture was centrifuged at 3600 × g for 5 min and the pellet was re-suspended in PBS to a desirable concentration, based on the optical density at 600 nm.

In vitro killing assay

MRSA or SAAP-148 were suspended in PBS or PBS containing 50% (v/v) blood plasma, 50% (v/v) eschar extract, 3% (v/v) bovine serum albumin (BSA; Thermo Fisher Scientific, Paisley, UK), 90% (v/v) DMEM (Gibco) or precipitated proteins from 50% (v/v) blood plasma or eschar extract. SAAP-148 was serially diluted 2-fold (0-30 nmol) and mixed with MRSA at a final concentration of 10^5 colony forming units (CFU)/ml. The final mixtures of 100 µl were briefly vortexed and incubated at 37°C. After 30 min of incubation, the samples were processed as previously described (Dijksteel et al., 2019b).

Ex vivo efficacy test

Ex vivo EWMs were inoculated with 10 µl of 10^7 CFU/mL MRSA for 1 h and topically exposed to 20 µL of PBS containing 0, 15, 30 and 60 nmol SAAP-148 for 1 h at 37 °C and 5% CO_2. The models were transferred to polypropylene vials containing a 7-mm metal bead and 1 ml of 0.05% (wt/v) sodium polyanethol sulfonate (SPS; Merck KGaA, Darmstadt, Germany) in PBS for homogenization using the TissueLyser set at 50 Hz for 4 min. Ten-fold serial dilutions of the homogenates were cultured on LB agar plates to quantify the number of viable bacteria after an overnight incubation at 37°C and 5% CO_2.

Cytotoxicity assay

In vitro

Human fibroblasts or keratinocytes were seeded (80,000/cm^2) in 96-well plates at 37°C and 5% CO_2. After 18 h, medium was discarded and cells were washed using PBS. Subsequently, 90 µl of DMEM followed by 10 µl of 2-fold serially diluted SAAP-148 in PBS (0-30 nmol) or 10% (v/v) Triton X-100 (Thermo Fisher Scientific) was added to the cells. After 1 h incubation at 37°C and 5% CO_2, the level of lactate dehydrogenase (LDH) in the media was determined as a measure for cytotoxicity using the non-radioactive cytotoxicity assay kit (Promega Corporation, Madison, USA), according to the manufacturer's instructions (Promega Corporation, n.d.). The absorbance of the samples was measured on a SpectraMax M2 (Molecular Devices, California, USA) at 490 nm. To determine the maximum LDH release, 10 µl of 10% (v/v) Triton X-100 was added to the surviving cells and the level of LDH was measured. The level of cytotoxicity was calculated as follows: [(experimental LDH release – spontaneous LDH release)/ (maximum LDH release – spontaneous LDH release)] × 100%.

Ex vivo

EWMs were exposed to 20 µL of PBS containing 0, 15, 30 and 60 nmol SAAP-148 or as negative control to 20 µl of 1% (v/v) Triton X-

100 for 1 h at 37°C and 5% CO_2. Subsequently, the models were washed in PBS and fixed in 1% (wt/v) paraformaldehyde in PBS for 2 h at 4°C, followed by an overnight incubation in 20% (wt/v) sucrose solution at 4°C while rotating. The samples were embedded in Tissue Tek O.C.T. compound (Sakura Finetek, Torrance, USA) to prepare 10 µm sections for LDH staining as previously described (Karim et al., 2019). LDH-stained cells were microscopically identified as a measurement for viable cells. To determine the level of cytotoxicity, we measured the area in the wound without LDH-stained cells using NIS Elements (Nikon Instruments Europe B.V., Amstelveen, the Netherlands).

Ex vivo wound healing

Ex vivo wound models were placed dermis down on stainless-steel grids for air-exposed culture at 37°C and 5% CO_2, as previously described (Coolen et al., 2008). The models were exposed to 20 µl of PBS containing 0, 15, 30 and 60 nmol SAAP-148, or 1% (wt/wt) SSD in cetomacrogol cream (Pharmacy of the Medical Center Alkmaar, Alkmaar, the Netherlands), twice a week. EWMs were cultured for two weeks and BWMs were cultured for three weeks to determine the effect of the peptide on re-epithelialization. Prior to exposure to SAAP-148, SSD or PBS, the culture medium was refreshed. Twenty-four hours prior to fixation of the *ex vivo* models in kryofix (50% ethanol and 3% PEG300), 20 µM 5-bromo-2-deoxyuridine (BrdU) was added to the culture medium.

Immunohistochemistry

Samples were processed for paraffin embedding and 5 µm sections were deparaffinized and rehydrated for HE and BrdU (B5002; Merck KGaA) staining, as previously described (Dijksteel et al., 2020). The newly formed epidermis (outgrowth) and the number of BrdU-positive cells/mm in the wounded area were microscopically determined using NIS Elements.

Statistical analysis

Statistical analysis was performed using SPSS version 24. For differences between groups the Kruskal-Wallis test followed by the Mann-Whitney-U test was used. To compare two related groups, the Wilcoxon signed rank sum test was used. Values of $p<0.05$ are considered significantly different.

RESULTS

Functional stability of SAAP-148 in 50% (v/v) blood plasma and eschar extract

Approximately 2.9 and 1 nmol SAAP-148 caused a 2.5-log reduction of MRSA in 100 µl of 50% (v/v) blood plasma and eschar extract, respectively, whereas 0.2 nmol SAAP-148 was sufficient to reach a 2.5-log reduction of MRSA in PBS environment (data not shown). To maximally reduce a bacterial count of 10^5 CFU/ml in 50% blood plasma and eschar extract, approximately 12.6 nmol SAAP-148 was necessary (data not shown). To determine if the bactericidal activity of SAAP-148 was dependent on the time that it resided in the biological environment, SAAP-148 was preincubated in 50% (v/v)
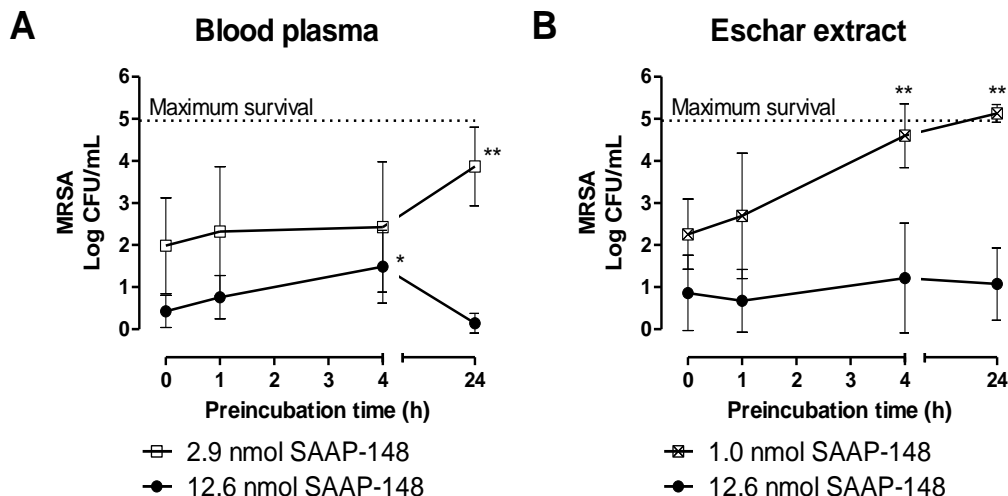
Figure 1. Functional stability of SAAP-148 in 50% blood plasma or eschar extract. Previously, results showed that 2.9 nmol and 1 nmol SAAP-148 caused a 2.5-log reduction of MRSA in 100 µL of 50% blood plasma or eschar extract, respectively, whereas 12.6 nmol SAAP-148 caused a maximum log-reduction of MRSA in both conditions (data not shown). Here we preincubated these SAAP-148 dosages in 50% blood plasma (A) or eschar extract (B) for 0, 1, 4 and 24 h. Subsequently, the preincubated SAAP-148 was added to 10^5 CFU/ml MRSA. Final mixtures of 100 µl were incubated at 37 °C and 5% CO_2 for 30 min. Results are expressed as the number of surviving bacteria in log10 (CFU/ml). Data represent the mean $\pm$ standard deviation (SD) of at least six independent experiments performed in triplicate. Statistical differences compared to SAAP-148 that had been pre-incubated for 0 h are indicated: *$p<0.05$; **$p<0.01$ (MWU).

blood plasma or eschar extract at a high (12.6 nmol) and low dosage (2.9 or 1 nmol). A 24 h preincubation of SAAP-148 at low dosages, capable to yield a 2.5-log reduction of MRSA, in 100 µl of 50% (v/v) blood plasma or eschar extract reduced the bactericidal efficacy of SAAP-148 considerably (Figure 1). In contrast, a 24 h preincubation period of 12.6 nmol SAAP-148 in 50% (v/v) blood plasma or eschar extract did not affect the bactericidal efficacy.

Effect of protease inhibition on SAAP-148's stability

The necessity for higher concentrations of SAAP-148 to reach equivalent bactericidal activity in biologically relevant environments as compared to PBS might be explained by proteolytic degradation of SAAP-148. Therefore, *in vitro* efficacy tests of SAAP-148 against MRSA were performed using 50% (v/v) blood plasma or eschar extract that had been incubated for 2 h with a cocktail of protease inhibitors or 8 mM EDTA to inhibit metalloproteases. Inhibition of proteolytic activity did not affect the bactericidal efficacy of SAAP-148 against MRSA in blood plasma and eschar extract (Figure 2).

## Bioactivity of SAAP-148 in relevant environments for wound healing

Interactions of SAAP-148 with proteins present in 50%

(v/v) blood plasma or eschar extract might reduce the bioavailability and thus affect the bactericidal efficacy of SAAP-148. Therefore, we precipitated the proteins in 50% (v/v) blood plasma or eschar extract and performed *in vitro* efficacy tests of SAAP-148 with the precipitated proteins dissolved in PBS. To achieve a 2-log reduction of MRSA in 100 µl of suspension, >20-fold higher dosages of SAAP-148 were required in the presence of the precipitated proteins as compared to PBS only (Figure 3). This effect is similar to the reduction found for 50% (v/v) blood plasma or eschar extract.

To assess the types of proteins that could be involved, we preincubated SAAP-148 with a collagen-elastin 3D matrix, Novomaix or 3% (v/v) BSA for 0, 1, 4 and 24 h. Subsequently, 10^5 CFU/mL MRSA was exposed to the preincubated SAAP-148 for 30 min. Figure 4A shows that the required dosage of SAAP-148 to induce bacterial killing in 100 µl of suspension gradually increased as the preincubation period of SAAP-148 with Novomaix increased from 0 to 24 h. An even stronger effect was seen as SAAP-148 was preincubated with 3% (v/v) BSA. This effect occurred instantly and remained until the 24 h preincubation period (Figure 4B).

Cytotoxicity of SAAP-148 for human fibroblasts and keratinocytes

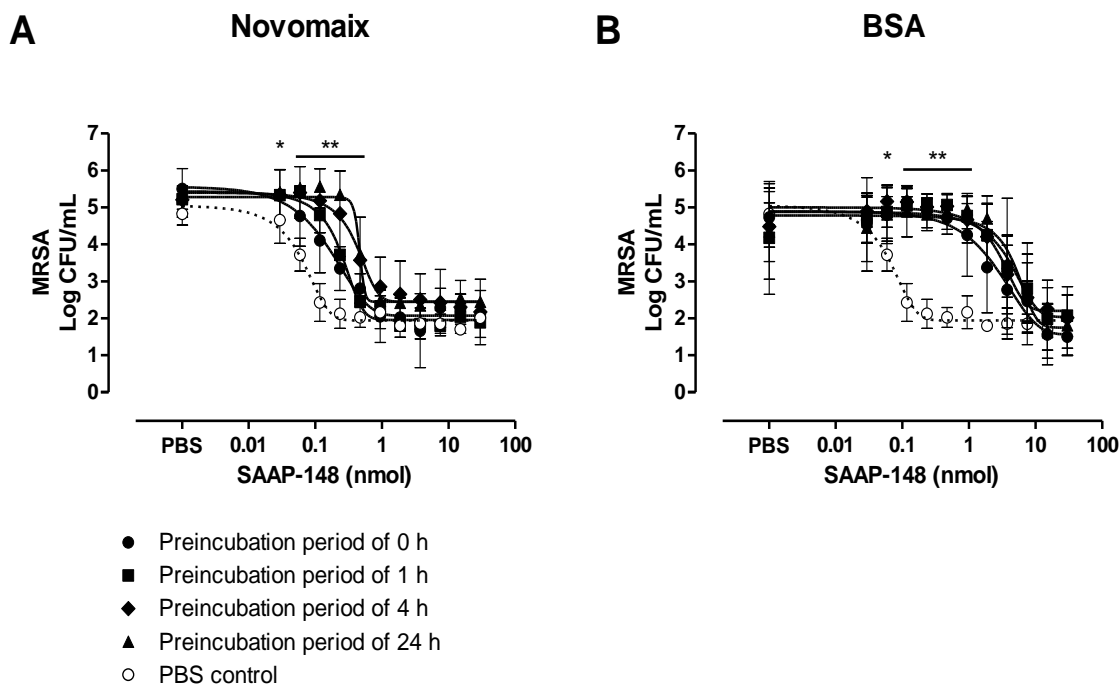Human fibroblasts and keratinocytes in 90 µl of medium were exposed to 10 µl of PBS containing increasing
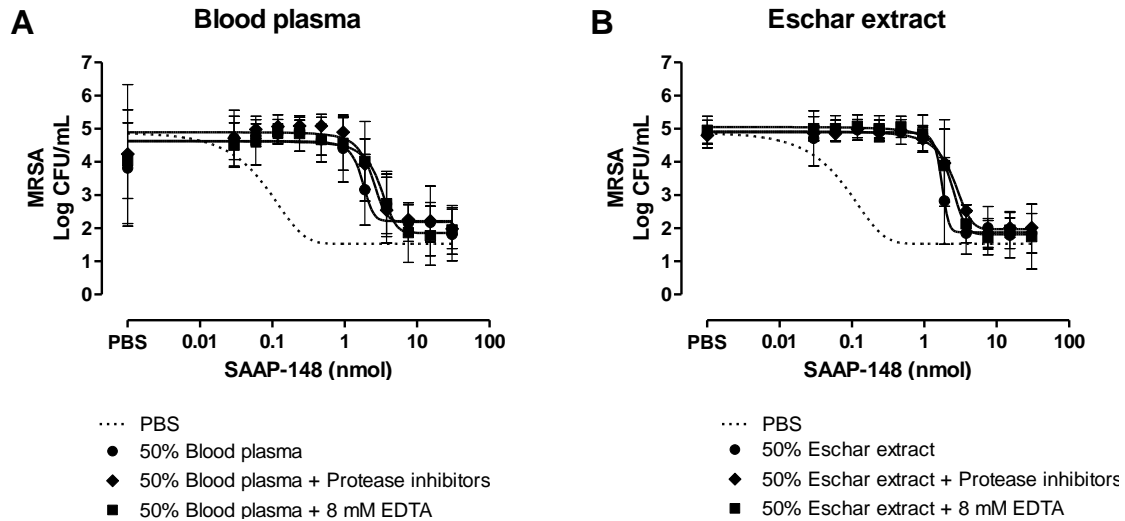
Figure 2. Protease inhibition and efficacy of SAAP-148.To inactivate proteolytic activity, a cocktail of protease inhibitors (EDTA-free) or 8 mM EDTA were added to 50% (v/v) blood plasma (A) or eschar extract (B) for 2 h. Subsequently, 10^5 CFU/mL MRSA were suspended herein and exposed to 2-fold serially diluted SAAP-148 (0-30 nmol). Final mixtures of 100 µl were incubated at 37°C and 5% CO_2 for 30 min. Results are expressed as the number of surviving bacteria in log10 (CFU/mL). Data represent the means ± SD of at least five independent experiments performed in duplicate. No statistical differences were detected compared to SAAP-148 in 50% (v/v) blood plasma or eschar extract (MWU; p>0.05).
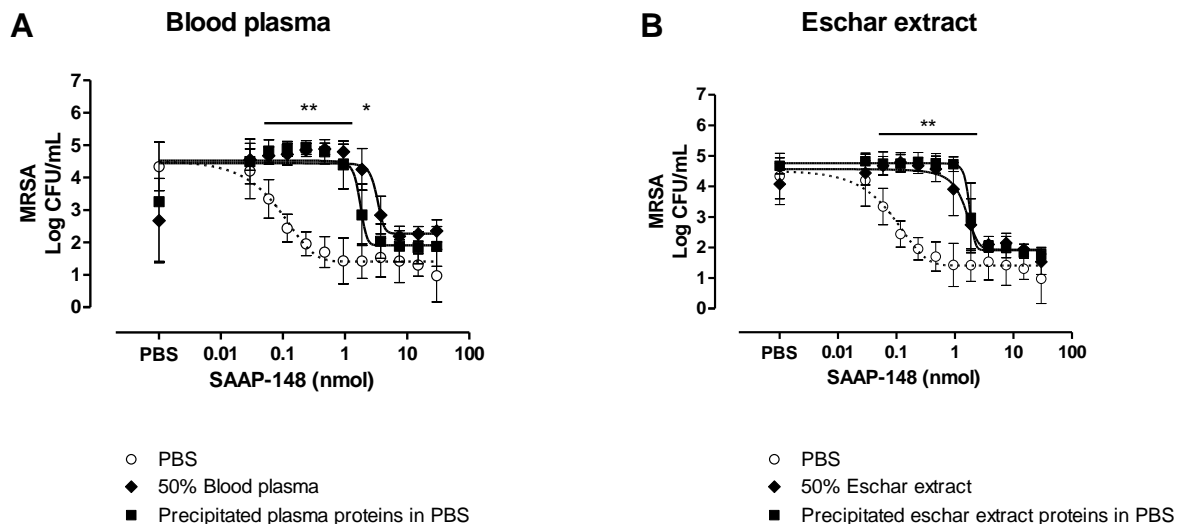
Figure 3. Effect of protein interactions on efficacy of SAAP-148. The proteins in 50% (v/v) blood plasma (A) or eschar extract (B) were precipitated using 90% (v/v) methanol, washed and dissolved in PBS. Approximately 10^5 CFU/mL MRSA were suspended herein and this was added to 2-fold serially diluted SAAP-148 (0-30 nmol). Final mixtures of 100 µL were incubated at 37 °C and 5% CO_2 for 30 min. As controls, *in vitro* efficacy tests of SAAP-148 against MRSA were performed in PBS, 50% blood plasma or eschar extract. Results are expressed as the number of surviving bacteria in log10 (CFU/mL). Data represent the means ± SD of six independent experiments performed in duplicate. Statistical differences compared to SAAP-148 in PBS are indicated: *p<0.05; **p<0.01 (MWU).

dosages of SAAP-148 for 1 h. Subsequently, we measured the amount of LDH, a cytosolic enzyme present in nearly all living cells, which is released into the culture medium as a result of cell damage. Figure 5A shows that SAAP-148 induced the release of cytosolic LDH for both human fibroblasts and keratinocytes in a

A   **Novomaix**   **B**   **BSA**

- ● Preincubation period of 0 h
- ■ Preincubation period of 1 h
- ◆ Preincubation period of 4 h
- ▲ Preincubation period of 24 h
- ○ PBS control

Figure 4. Functional stability of SAAP-148 in the presence of Novomaix or BSA. Two-fold serially diluted SAAP-148 were preincubated in 100 µL of PBS and in the presence of a collagen-elastin 3D matrix, Novomaix (A) or 3% (v/v) BSA (B) for 0, 1, 4 and 24 h. Subsequently, the preincubated SAAP-148 (0-30 nmol) was mixed with 10^5 CFU/ml MRSA. Final mixtures of 100 µl were incubated at 37°C and 5% CO_2 for 30 min. Results are expressed as the number of surviving bacteria in log10 (CFU/mL). Data represent the means $\pm$ SD of at least five independent experiments performed in duplicate. Statistical differences of SAAP-148 that had been incubated for 24 h versus SAAP-148 in PBS are indicated: *p<0.05; **p<0.01, (MWU).

dose-dependent manner. Notably, a SAAP-148 dosage of only 0.23 nmol in 100 µl of 90% (v/v) DMEM resulted in 50% LDH release. To determine the level of cytotoxicity of SAAP-148 for human skin cells in their natural biological environment, *ex vivo* EWMs were exposed to 20 µl of PBS containing various dosages of SAAP-148 and – as positive control - Triton X-100 for 1 h. Subsequently, the viable cells were identified by staining tissue sections for LDH. SAAP-148 did not penetrate through the whole thickness of the skin sample (data not shown) and therefore the level of cytotoxicity was determined using the area in the sample without LDH-stained cells. Unlike Triton X-100, SAAP-148 dosages up to 60 nmol/model did not eliminate the LDH containing cells in the excision wounds (Figure 5B and C).

To determine the therapeutic index of SAAP-148, the bactericidal efficacy of this peptide was determined at the same conditions as the cytotoxicity assays. SAAP-148 eradicated MRSA in a dose-dependent manner *in vitro* and approximately 0.23 nmol SAAP-148 in 100 µl of medium resulted in a 3-log reduction of MRSA (Figure 6A). In *ex vivo* EWMs, SAAP-148 was equally potent against MRSA at 15, 30 and 60 nmol/model (Figure 6B). Approximately 15 nmol of SAAP-148 was sufficient to achieve a 3-log reduction of MRSA. Hence, SAAP-148

was effective against MRSA at the same dosage that induced cytotoxicity in 2D culture, resulting in a therapeutic index of 1. In contrast, the lowest tested dosages of 15 nmol SAAP-148 effectively eradicated MRSA in *ex vivo* excision wound models, whereas the highest tested dosage of 60 nmol SAAP-148 was not sufficient to induce 50% cytotoxicity *ex vivo*. This indicates a therapeutic index of at least 4.

Effect of SAAP-148 on wound healing

To determine the effect of SAAP-148 on wound healing, we exposed *ex vivo* EWMs and BWMs to 20 µL of PBS containing 0, 15, 30 and 60 nmol SAAP-148 or 1% (wt/wt) SSD. Subsequently, we measured the newly formed epidermis (re-epithelialization) and the number of proliferative cells in that area. A SAAP-148 dose of 60 nmol/model showed no statistically significant negative effects on the re-epithelialization of EWMs (Figure 7A) and BWMs (Figure 7B). In addition, SAAP-148 did not affect the number of proliferating cells in the EWMs and BWMs (data not shown). Contrarily, exposure to SSD resulted in little to no re-epithelialization and proliferating cells in both types of wound models.

A

C

B
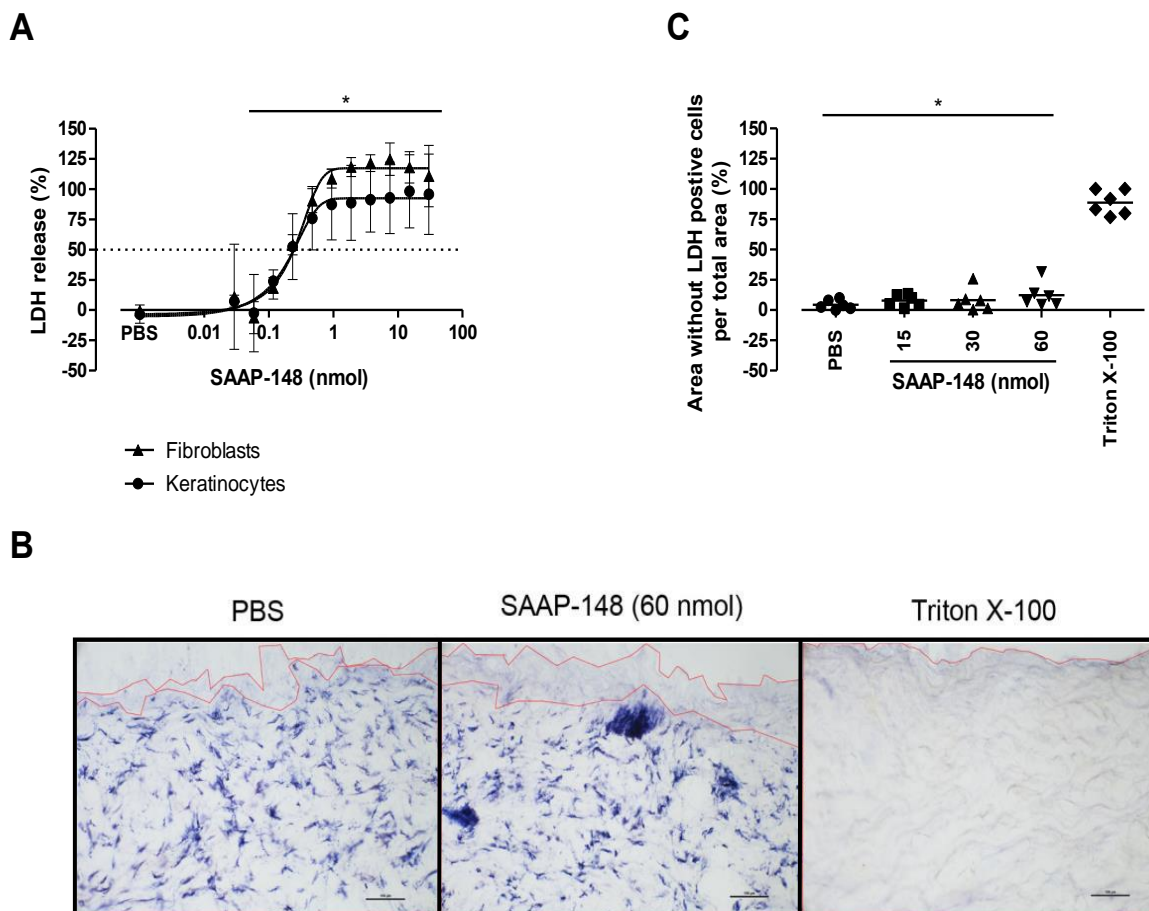
Figure 5. *In vitro* and *ex vivo* cytotoxicity of SAAP-148 for human skin cells. (A) The *in vitro* cytotoxicity of SAAP-148 for human fibroblasts and keratinocytes was determined by exposing these cells to 2-fold serially diluted SAAP-148 (0-30 nmol). Final mixtures of 100 µl were incubated at 37°C and 5% CO_2 for 1 h. Subsequently, the amount of the LDH enzyme in culture medium and of remaining viable cells was measured. Results are expressed as the LDH-release of total LDH-release in percentages. The dotted line indicates the dosage required to induce 50% cytotoxicity. Data represent the means ± SD of six independent experiments performed in triplicate. Statistical differences compared to the PBS-control are indicated: *$p < 0.05$ (Wilcoxon-S-R). (B/C) *Ex vivo* EWMs were exposed to 20 µl of PBS containing 0, 15, 30 and 60 nmol SAAP-148 or 1% (v/v) Triton X-100 for 1 h. (B) The viable cells were identified by staining for the cytoplasmic enzyme LDH (blue). The area without LDH positive cells is surrounded by red lines. Scale bars represent 100 µm. (C) Results are expressed as the area without LDH positive cells relative to the total area at two random locations in the upper dermal layer of excision wounds. Data represent the means of six independent experiments performed in duplicate. Statistical differences compared to the Triton X-100 exposed models are indicated: *$p < 0.05$ (Wilcoxon-S-R).

DISCUSSION

In the current study, we tried to elucidate why higher concentrations of SAAP-148 in 50% (v/v) blood plasma or eschar extract are necessary to reach a similar bactericidal effect as in PBS (Dijksteel et al., 2019a). We have shown that preincubation of SAAP-148 in these biological relevant environments significantly reduced the bactericidal efficacy of this peptide against MRSA. This suggested a reduced bioavailability of SAAP-148 that could have been caused by proteolytic degradation and/or binding of SAAP-148. The bactericidal efficacy of several AMPs including LL-37 is reduced by proteolytic activity in the presence of blood plasma (McCrudden et al., 2014; Sieprawska-Lupa et al., 2004; Thwaite et al., 2006). Previously, naturally occurring and synthetic AMPs were found to be susceptible to protease activity (Moncla et al., 2011), whereas other AMPs displayed a reduced bioactivity due to interactions with albumin or NaCl (Björn et al., 2016; Svenson et al., 2007). Similar to some synthetic AMPs (Shin et al., 2010), SAAP-148 was not degraded by proteases present in blood plasma or eschar extract.

The reduced bioactivity of SAAP-148 seemed related to protein interactions, resulting in a reduced bioavailability of SAAP-148 to combat MRSA.

A

B
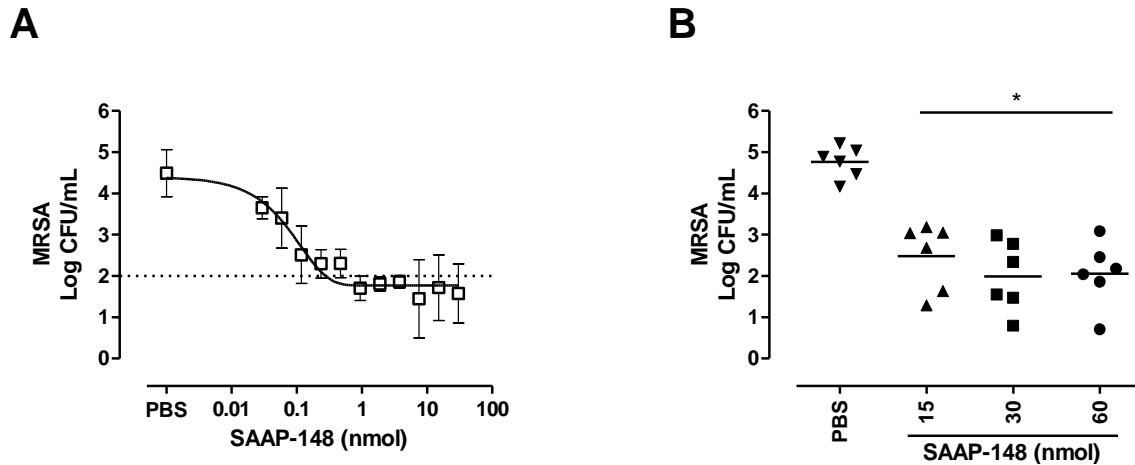
Figure 6. Efficacy of SAAP-148 against MRSA.
(A) *In vitro* efficacy tests of SAAP-148 (0-30 nmol) were performed to determine the effective dosage required to combat 10^5 CFU/ml MRSA. Final mixtures were incubated in 100 µl of 90% (v/v) DMEM for 1 h. Data represent the means ± SD of three independent experiments performed in duplicate. B: Approximately, 10^5 CFU MRSA in *ex vivo* EWMs were exposed to 20 µl of PBS containing 0, 15, 30 and 60 nmol SAAP-148 for 1 h. Data represent the means of six independent experiments performed in duplicate. Results are expressed as the number of surviving bacteria in log10 (CFU/ml). Statistical differences compared to the PBS-exposed models are indicated: *p<0.05 (Wilcoxon-S-R).

A   **EWM**

B   **BWM**
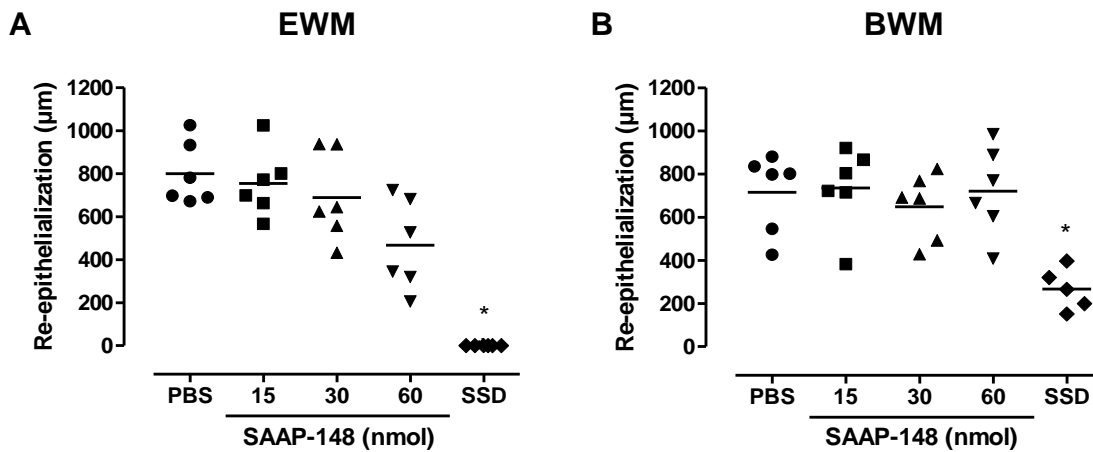
Figure 7. Effect of SAAP-148 on wound healing. *Ex vivo* wound models were exposed twice weekly to 20 µl of PBS containing 0, 15, 30 and 60 nmol SAAP-148 or 1% (wt/wt) SSD. EWMs (A) were cultured for two weeks, whereas BWMs (B) were cultured for three weeks. Subsequently, the newly formed epidermis and the number of proliferative cells in this area were measured in microscopic sections. Results are expressed as the re-epithelialization in µm. Data represent the means of six independent experiments performed in duplicate. Statistical differences compared to the SAAP-148- or PBS-exposed models are indicated: *p<0.05 (Wilcoxon-S-R).

Our findings are in agreement with those reported by de Breij et al. (2018) demonstrating a concentration dependent reduction in the bactericidal efficacy of SAAP-148 in the presence of high density lipoproteins or human serum albumin.

It is striking that the presence of BSA instantly reduced the bactericidal efficacy of SAAP-148 a 100-fold, whereas a 24 h incubation period of SAAP-148 with the collagen-elastin 3D matrix, Novomaix, was required to significantly reduce the bactericidal efficacy of SAAP-148. This suggests that the presence of blood-derived rather than skin-derived proteins within the eschar extract was predominantly responsible for the reduced bactericidal efficacy of SAAP-148. In addition, the discrepancy

between instant albumin response and the slower plasma response on the reduction of the bactericidal effect might be explained by the presence of other proteins in blood plasma. Hence, blood protein interactions with SAAP-148 may have reduced the bioavailability of the peptide and this remains a challenge for the development of SAAP-148 as a topical antimicrobial agent for burns.

The last decade, the bactericidal efficacy of AMPs against pathogens has improved but their effects on mammalian cells have not been fully investigated. Therefore, we included cytotoxicity studies of SAAP-148 for human skin cells. SAAP-148 did display some cytotoxic effects on human fibroblasts and keratinocytes in *in vitro* cultures but not in *ex vivo* skin. This suggests that in 3D skin, cells are protected by the extracellular matrix. For a set of different AMPs, the *in vitro* therapeutic index was determined, whereby the highest therapeutic index was found for cecropins and the smallest for melittin (Bacalum and Radu, 2015). Based on our findings, the *in vitro* therapeutic index of SAAP-148 was lower than for melittin. Nonetheless, the therapeutic index of SAAP-148 for *ex vivo* human skin models was at least 4-times higher than for *in vitro* skin cells. Hence, the difference between the *in vitro* and *ex vivo* findings highlights the importance to evaluate the bactericidal efficacy and safety of AMPs such as SAAP-148 in models that better simulate the clinical situation.

Several AMPs such as LL-37, OA-GL12 and GE33 have the ability to promote wound healing (Huang et al., 2014; Ramos et al., 2011; Song et al., 2019; Steinstraesser et al., 2008). Therefore, we determined the effect of SAAP-148 on wound healing. SAAP-148 showed no negative effects on the re-epithelialization of *ex vivo* excision wounds and burn wounds. The 'gold standard' for treatment of burn wound infections, SSD, induced cellular toxicity, which resulted in a delayed wound closure. Since SAAP-148 had no effects on wound healing while it was effective against resistant bacteria, SAAP-148 could be a potential alternative for SSD as a topical antimicrobial strategy for burns.

To improve the bioavailability of AMPs, different approaches can be used such as the introduction of D-amino acids into the AMPs' amino acid sequence, cyclization, amidation or acetylation of the thermal regions (Chu et al., 2013; Gentilucci et al., 2010; Strömstedt et al., 2009). These approaches, however, are designed to improve the stability of AMPs against proteolytic degradation. Chemical modifications, such as tryptophan end-tagging, the introduction of the non-natural amino acid β-naphtlylalanine or the replacement of tryptophans and histidines in Trp- and His-rich AMPs could result in an improved salt and serum stability (Chu et al., 2013; Pfalzgraff et al., 2018). Also, amino acid substitution to decrease the net positive charge of the peptide within a certain range could result in less cytotoxicity without effecting the antimicrobial efficacy (Jiang et al., 2008; Xu et al., 2020).

Alternatively, SAAP-148 might be protected against unwanted protein and cell binding using drug delivery systems, such as hydrogels, polylactic-co-glycolic acid nanoparticles or lipid-based encapsulation systems (Meikle et al., 2017; Xie et al., 2015). Formulation of SAAP-148 might not only result in an improved bioavailability of the peptide (Haisma et al., 2016), but also in an improved delivery of the AMPs to deeper layers of the skin.

Conclusions

In the presence of blood plasma, blood proteins or skin proteins, higher concentrations of SAAP-148 were required to reach the same bactericidal effects as in PBS. The reduced bioavailable concentrations of SAAP-148 were not caused by proteolytic degradation but presumably by protein binding. Furthermore, in contrast to isolated human skin cell cultures, relatively high concentrations of SAAP-148 did not display cytotoxic effects in *ex vivo* excision wounds and burn wounds and allowed undisturbed epithelialization while retaining bactericidal activity.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ABBREVIATIONS

AMPs, antimicrobial peptides; **BrdU,** 5-bromo-2-deoxyuridine; **BSA,** bovine serum albumin; **CFU,** colony forming units; **DMEM,** Dulbecco's Modified Eagle Medium; **DNA,** deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; **EWM,** excision wound model; FCS, fetal calf serum; LB, Luria Bertani; **LDH,** lactate dehydrogenase; **MRSA,** methicillin resistant *Staphylococcus aureus*; **PBS,** phosphate-buffered saline; **P/S,** penicillin/streptomycin; **RPMI,** Roswell Park Memorial Institute; **SD,** standard deviation; **SPS,** sodium polyanethol sulfonate; **SSD,** silver sulfadiazine.

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