Antibacterial activity and therapeutic efficacy of Fl-P<sub>R</sub>P<sub>R</sub>P<sub>L</sub>-5, a cationic amphiphilic polyproline helix, in a mouse model of staphylococcal skin infection

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Abstract: The antibacterial activities and therapeutic efficacy of the cationic, unnatural proline-rich peptide Fl-P<sub>R</sub>P<sub>R</sub>P<sub>L</sub>-5 were evaluated against multidrug-resistant <i>Staphylococcus aureus</i> in a mouse model of skin infection. Fl-P<sub>R</sub>P<sub>R</sub>P<sub>L</sub>-5 showed potent activity against all clinical isolates of <i>S. aureus</i> tested, including methicillin- and vancomycin-resistant <i>S. aureus</i> (MRSA and VRSA, respectively). Fl-P<sub>R</sub>P<sub>R</sub>P<sub>L</sub>-5 was also superior in clearing established in vitro biofilms of <i>S. aureus</i> and <i>Staphylococcus epidermidis</i>, compared with the established antimicrobials mupirocin and vancomycin. Additionally, topical treatment of an MRSA-infected wound with Fl-P<sub>R</sub>P<sub>R</sub>P<sub>L</sub>-5 enhanced wound closure and significantly reduced bacterial load. Finally, 0.5% Fl-P<sub>R</sub>P<sub>R</sub>P<sub>L</sub>-5 significantly reduced the levels of the inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β) in wounds induced by MRSA skin infection. In conclusion, the results of this study suggest the potential application of Fl-P<sub>R</sub>P<sub>R</sub>P<sub>L</sub>-5 in the treatment of staphylococcal skin infections.

Keywords: antimicrobial peptides, <i>Staphylococcus aureus</i>, biofilms, anti-inflammatory, skin infection

Introduction

<i>Staphylococcus aureus</i>, a common and virulent pathogen, is the leading cause of nosocomial wound infections.<sup>1–3</sup> Virulence factors, toxins, and exoproteins secreted by multidrug-resistant strains of <i>S. aureus</i> play a major role in evading host immune response, leading to persistent infection, prolonged inflammation, and delayed wound healing.<sup>4–6</sup> Furthermore, staphylococcal skin infections often lead to invasive infections that ultimately result in septicemia.<sup>7,8</sup> Recently, superficial cutaneous bacterial infections caused by biofilm-producing <i>Staphylococci</i> multidrug-resistant strains have become an emerging clinical problem often associated with treatment failure, even with topical drugs of choice, such as mupirocin and fusidic acid.<sup>9–11</sup> Hence, there is an urgent need for novel antimicrobials that can be used to treat cutaneous staphylococcal infections successfully and overcome the antibiotic resistance associated with biofilm-producing <i>Staphylococci</i> spp. One potential alternative approach is the development of antimicrobial peptides as novel therapeutic agents.<sup>12</sup> Antimicrobial peptides with anti-inflammatory properties in addition to potent, broad-spectrum antimicrobial activity might form a promising approach to the treatment of staphylococcal skin infections.<sup>12,13</sup>
In a previous study, we designed a cationic, unnatural proline-rich antimicrobial peptide (Fl-P$^R_5$$^R_5$P$^L_4$) with intrinsic cell penetrating and antibacterial activities (Figure 1). Fl-P$^R_5$$^R_5$P$^L_4$ adopts an amphiphilic polyproline type II helix with a net positive charge and exhibits broad-spectrum activity against various Gram-positive and Gram-negative pathogens, including multidrug-resistant strains of S. aureus. However, we recently synthesized a polyproline type II helix with an additional repeat of cationic and hydrophobic moieties, which we call Fl-P$^R_5$$^R_5$P$^L_5$ (Figure 1). We previously demonstrated that Fl-P$^R_5$$^R_5$P$^L_5$ shows enhanced cell penetration and higher antimicrobial activity than Fl-P$^R_5$$^R_5$P$^L_4$. In the current study, we tested the efficacy of Fl-P$^R_5$$^R_5$P$^L_5$ against clinical isolates of multidrug-resistant S. aureus and evaluated its antibiofilm activity. We also tested its in vivo antimicrobial and anti-inflammatory properties in a mouse model of methicillin-resistant S. aureus (MRSA) skin infection.

Materials and methods

Bacterial strains and reagents

Staphylococcus strains used in this study are presented in Table 1. Mupirocin was purchased from Applichem GmbH (Darmstadt, Germany), and vancomycin hydrochloride from Gold Biotechnology, Inc (Olivette, MO, USA). Mueller–Hinton broth was purchased from Sigma-Aldrich from Gold Biotechnology, Inc (Olivette, MO, USA). Tryptic soy broth, tryptic soy agar, and mannitol salt agar were purchased from BD (Franklin Lakes, NJ, USA).

Synthesis of Fl-P$^R_5$$^R_5$P$^L_5$

The unnatural amino acids needed for the synthesis of Fl-P$^R_5$$^R_5$P$^L_5$ were synthesized, as previously described. The peptide was synthesized on the Rink amide resin using a fluorenylmethoxycarbonyl- (Fmoc)-based strategy with 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluorophosphate (HATU) as the coupling agent. At the conclusion of the synthesis of the peptide, the resin was treated with N-hydroxysuccinimide fluorescein. The resin-bound fluorescein-modified peptide was cleaved from the resin with a trifluoroacetic acid cocktail; the resulting material was purified to homogeneity by reverse-phase high-performance liquid chromatography, and was then characterized by mass spectrometry.

Antibacterial assays

The broth micro-dilution method was used to determine the minimum inhibitory concentration (MIC) of Fl-P$^R_5$$^R_5$P$^L_5$, as per the guidelines outlined by the Clinical and Laboratory Standards Institute (CLSI). Fl-P$^R_5$$^R_5$P$^L_5$ was incubated with bacteria for 16 hours at 37°C, and the lowest concentration of peptide capable of inhibiting visible growth of bacteria by visual inspection was recorded as MIC. The minimum bactericidal concentration of the peptide was determined by sub-culturing the wells where no growth was observed onto tryptic soy agar plates. The plates were incubated for 24 hours, and the concentration where ≥99.9% reduction in bacterial colony-forming units was observed was recorded as the minimum bactericidal concentration.

Biofilm assay

The ability of Fl-P$^R_5$$^R_5$P$^L_5$ and control antibiotics (mupirocin and vancomycin) to disrupt established biofilms of S. aureus (American Type Culture Collection [ATCC] 6538) and S. epidermidis (ATCC 35984) was evaluated by using the microtiter dish biofilm formation assay. Briefly, bacteria were grown in 96-well tissue-culture-treated plates in tryptic soy broth supplemented with 1% glucose for 24 hours at 37°C. The media were removed, and plates were washed four times with phosphate-buffered saline, and the adherent biofilms attached to the wells were treated with the concentration mentioned in the figure of peptide and control antibiotics. After 24 hours, plates were again washed with water, and biofilms were stained with 0.1% (weight/volume) crystal violet for 30 minutes at room temperature. The plates were then air dried, and biofilms were solubilized using 95% ethanol. Intensity of crystal violet was measured using a microplate reader (Bio-Tek Instruments Inc, Winooski, VT, USA) at the optical density of 595 nm.

Figure 1 Structure of the cationic amphiphilic antimicrobial peptides containing unnatural proline-based amino acids.
Table 1 Antimicrobial activity of Fl-P<sub>R</sub>P<sub>R</sub>P<sub>L</sub>-5 against clinical isolates of *Staphylococcus aureus*

<table>
<thead>
<tr>
<th>Staphylococcus strain name</th>
<th>Origin</th>
<th>Molecular typing</th>
<th>Phenotypic properties</th>
<th>MIC/MBC (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NARSA ID</td>
<td>Alternate designation</td>
<td>SCCmec type</td>
<td>spa type</td>
<td></td>
</tr>
<tr>
<td>NRS382 USA100</td>
<td>US</td>
<td>II</td>
<td>TJMMDGMK</td>
<td>Resistant to ciprofloxacin, clindamycin, erythromycin, and mecillin</td>
</tr>
<tr>
<td>NRS383 USA200</td>
<td>US</td>
<td>II</td>
<td>WGKAKOMQGQ</td>
<td>Resistant to ciprofloxacin, clindamycin, erythromycin, gentamicin, and mecillin</td>
</tr>
<tr>
<td>NRS384 USA300</td>
<td>US</td>
<td>IV</td>
<td>YHGMBQBL</td>
<td>Resistant to erythromycin, mecillin, and tetracycline</td>
</tr>
<tr>
<td>NRS386 USA700</td>
<td>US</td>
<td>IV</td>
<td>UJGFMG</td>
<td>Resistant to erythromycin and mecillin</td>
</tr>
<tr>
<td>NRS1077</td>
<td>US</td>
<td>II</td>
<td>TMBDMGMK</td>
<td>Resistant to vancomycin</td>
</tr>
<tr>
<td>VRS55</td>
<td>US</td>
<td>-</td>
<td>-</td>
<td>Biofilm-producing clinical isolate</td>
</tr>
<tr>
<td>ATCC 6358</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S. epidermidis NRS-101</td>
<td>US</td>
<td>-</td>
<td>-</td>
<td>Biofilm-producing clinical isolate; resistant to mecillin and gentamicin</td>
</tr>
</tbody>
</table>

**Abbreviations:** NARSA, Network on Antimicrobial Resistance in *Staphylococcus aureus*; ID, identification; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; ATCC, American Type Culture Collection; spa, staphylococcal protein A; SCC, staphylococcal cassette chromosome; –, not available.

Mice infection

Murine MRSA skin infection study was performed, as described before.<sup>20</sup> Briefly, groups of mice (five mice per group) were infected intradermally with 1.65×10<sup>7</sup> colony-forming units of MRSA USA300 and were left for 48 hours before an open wound formed at the injection site. Mice were divided into three groups, and each group of mice was treated with either 0.5% Fl-P<sub>R</sub>P<sub>R</sub>P<sub>L</sub>-5, 2% mupirocin, or vehicle alone (petroleum jelly). All groups of mice were treated once a day for 4 days. Exactly 24 hours after the last treatment, mice were euthanized, and the area around the wound was slightly swabbed with 70% ethanol. Wound area (1 cm<sup>2</sup>) was precisely excised, homogenized, and plated onto mannitol salt agar plates to count viable bacterial colony-forming units. The animal care and all experiments were performed in accordance with the guidelines approved by Purdue University Animal Care and Use Committee (PACUC).

Quantifying inflammatory cytokines

Skin homogenates obtained from the MRSA-infected wounds were centrifuged, and the supernatants were used to quantify the levels of inflammatory cytokines, including tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), interleukin-1 beta (IL-1β), and monococyte chemoattractant protein-1. Duo-set enzyme-linked immunosorbent assay kits (R&D Systems, Inc, Minneapolis, MN, USA) were employed to detect cytokine levels, and the experiment was carried out using the manufacturer’s protocol.<sup>21</sup>

Statistical analyses

Statistical analyses were assessed by Graph Pad Prism 6.0 (Graph Pad Software, La Jolla, CA, USA). P-values were calculated by the two-tailed Student’s t-test. P-values of <0.05 were considered as significant.

Results and discussion

Fl-P<sub>R</sub>PP<sub>L</sub>-5 has potent activity against clinical isolates of *S. aureus*

The antimicrobial activity of Fl-P<sub>R</sub>P<sub>R</sub>P<sub>L</sub>-5 was evaluated against a panel of clinical *S. aureus* isolates (Table 1). Fl-P<sub>R</sub>P<sub>R</sub>P<sub>L</sub>-5 showed bactericidal activity against this panel of multidrug-resistant *S. aureus* strains that are resistant to mecillin, gentamicin, mupirocin, vancomycin, clindamycin, and erythromycin. Fl-P<sub>R</sub>P<sub>R</sub>P<sub>L</sub>-5 was also active against MRSA USA300, which has been linked to the majority of skin and soft tissue infections present in the US.<sup>22</sup> Fl-P<sub>R</sub>P<sub>R</sub>P<sub*L</sub>-5 showed bactericidal activity against all tested strains of *S. aureus*, regardless of their antibiotic resistance, with MICs ranging from 8–16 µM (Table 1).

Fl-P<sub>R</sub>P<sub>R</sub>P<sub>L</sub>-5 is effective in clearing preformed biofilms

Bacterial biofilms play a major role in bacterial resistance. They often impede antimicrobial penetration, leading to treatment failures that increase the rate of emergence of bacterial resistance.<sup>9,23</sup> Therefore, we evaluated the effect of Fl-P<sub>R</sub>P<sub>R</sub>P<sub*L</sub>-5 on preformed biofilms. As shown in Figure 2, treatment with Fl-P<sub>R</sub>P<sub>R</sub>P<sub*L</sub>-5 and control antibiotics (mupirocin and vancomycin) significantly reduced the masses of preformed biofilms generated by biofilm-forming strains of *S. aureus* and *S. epidermidis*. Fl-P<sub>R</sub>P<sub>R</sub>P<sub*L</sub>-5 was found to be significantly superior to mupirocin and vancomycin in reducing biofilm mass. Fl-P<sub>R</sub>P<sub>R</sub>P<sub*L</sub>-5, at twice the MIC reduced the mass of the *S. aureus* biofilm, by more than 50%. Mupirocin and vancomycin reduced the mass of the established biofilm by only 25%, even at 32 times their respective MICs (Figure 2A). Similarly, Fl-P<sub>R</sub>P<sub>R</sub>P<sub*L</sub>-5, at
a concentration 16 times its MIC, significantly reduced the mass of a pre-established *S. epidermidis* biofilm by approximately 40%, whereas mupirocin and vancomycin, at 128 times their respective MICs, reduced the mass of the biofilm by approximately 10% and 20%, respectively (Figure 2B). These results suggest that Fl-P₅R₆P₇L₅ exhibits substantially more potent activity against staphylococcal biofilms than the traditional antimicrobials mupirocin and vancomycin.

**Fl-P₅R₆P₇L₅ enhances wound closure and reduces bacterial load**

Confident in the broad-spectrum antimicrobial activity and potent antibiofilm properties of Fl-P₅R₆P₇L₅, we tested its in vivo anti-staphylococcal activity in a mouse model of MRSA skin infection. Three groups of BALB/C mice were intradermally infected with MRSA USA300. The open wound that formed 2 days after infection was treated topically with vehicle alone (petroleum jelly), 2% mupirocin, or 0.5% Fl-P₅R₆P₇L₅ once a day for 4 days. As shown in Figure 3A, topical treatment with 0.5% Fl-P₅R₆P₇L₅ for 4 days considerably enhanced the rate of wound closure compared with the control and 2% mupirocin treatment groups. However, the mean bacterial count was significantly reduced in both the mupirocin and Fl-P₅R₆P₇L₅ treatment groups, compared with the control group (**P values of ≤0.05**). As shown in Figure 3B, the groups treated with 2% mupirocin had the highest reduction in
bacterial count (95%) followed by 0.5% Fl-P\textsuperscript{R_R_P_L} (75%). These results suggest that topical application of 0.5% Fl-P\textsuperscript{R_R_P_L} exerts a positive effect on the wound healing process and reduces the bacterial load in MRSA-infected wounds.

**Fl-P\textsuperscript{R_R_P_L} reduces inflammatory cytokines induced by MRSA infection**

Excess inflammation, which is often associated with *S. aureus* skin infections, increases the clinical severity of the infection.\textsuperscript{4–6} Therefore, we tested the immunomodulatory properties of Fl-P\textsuperscript{R_R_P_L} in a mouse model of MRSA skin infection. The effect of Fl-P\textsuperscript{R_R_P_L} on levels of the inflammatory cytokines TNF-\(\alpha\), IL-6, IL-1\(\beta\), and monocyte chemoattractant protein-1 was measured in the infected wounds using enzyme-linked immunosorbent assays. As shown in Figure 4, Fl-P\textsuperscript{R_R_P_L} (0.5%) significantly reduced the levels of TNF-\(\alpha\), IL-6, and IL-1\(\beta\). In contrast, 2% mupirocin significantly reduced the level of only IL-6. These results indicate that, in addition to having potent antimicrobial activity, Fl-P\textsuperscript{R_R_P_L} has considerably higher anti-inflammatory properties than the traditional antimicrobial mupirocin.

**Conclusion**

In summary, we conclude that Fl-P\textsuperscript{R_R_P_L}, a new antimicrobial peptide, possesses anti-staphylococcal and antibiofilm activities against multidrug-resistant clinical isolates of *S. aureus*. In addition, this peptide enhances wound closure and reduces bacterial load in MRSA-infected wounds. Fl-P\textsuperscript{R_R_P_L} reduced levels of the inflammatory cytokines TNF-\(\alpha\), IL-6, and IL-1\(\beta\) in wounds induced by MRSA infection. The anti-inflammatory properties of Fl-P\textsuperscript{R_R_P_L} might also have a beneficial effect on the healing of chronic and diabetic wounds.\textsuperscript{23–27} However, additional studies are needed to determine the exact mechanisms involved in the wound healing process. In conclusion, our study suggests that Fl-P\textsuperscript{R_R_P_L} might constitute a novel therapeutic option for the treatment of cutaneous staphylococcal skin infections. The current study also forms a strong platform for the further exploration of the immunomodulatory activities of antimicrobial peptides as part of a novel strategy for the treatment of staphylococcal skin infections.

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Disclosure
The authors report no conflicts of interest in this work.

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