

Efficacy of short novel antimicrobial and anti-inflammatory peptides in a mouse model of methicillin-resistant *Staphylococcus aureus* (MRSA) skin infection

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Abstract: The therapeutic efficacy of two novel short antimicrobial and anti-inflammatory peptides (RR and RRIKA) was evaluated in a mouse model of staphylococcal skin infection. RR (2%) and RRIKA (2%) significantly reduced the bacterial counts and the levels of proinflammatory cytokines, tumor necrosis factor (TNF)- α , and interleukin (IL)-6, in methicillin-resistant *Staphylococcus aureus* USA 300-0114 skin lesions. Furthermore, the combined therapy of RRIKA (1%) and lysostaphin (0.5%) had significantly higher antistaphylococcal and anti-inflammatory activity compared to monotherapy. This study supports the potential use of these peptides for topical treatment of methicillin-resistant *Staphylococcus aureus* skin infections.

Keywords: antimicrobial peptides, MRSA, lysostaphin, skin infection, mice

Introduction

Bacterial infections, especially those caused by *Staphylococcus aureus*, are the most significant complication encountered in the management of wounds.¹ Furthermore, multidrug-resistant *S. aureus* strains and their secreted toxins are responsible for interfering with the wound-healing process and creating portals of entry for systemic complications in affected patients.² With the increasing incidence of staphylococcal resistance to topical antimicrobials, such as mupirocin and fusidic acid,³⁻⁵ there is a pressing need to develop novel antimicrobials and new approaches to circumvent this burgeoning problem. Recently, there has been increased interest in the development of antimicrobial peptides (AMPs) as novel therapeutics, due to their high potency, broad spectrum of activity, and reduced potential for resistance development.^{6,7} In addition to the potent bactericidal activity of AMPs, the recognized anti-inflammatory response of certain AMPs should be an advantage in the treatment of *S. aureus* skin infections.⁸

In a recent study, we described two novel short peptides – RR (WLRRIKAWLRR) and RRIKA (WLRRIKAWLRRIKA) – with potent bactericidal activity in vitro against multiple clinical isolates of methicillin-resistant *S. aureus* (MRSA).⁶ In particular, the peptides were active against the highly virulent MRSA USA 300-0114, a community-associated strain responsible for outbreaks of staphylococcal skin and soft-tissue infections in the US.⁹ Moreover, RR and RRIKA were superior in reducing adherent biofilms of both *S. aureus* and *Staphylococcus epidermidis* when compared to conventional antibiotics. Furthermore, both RR and RRIKA enhanced the antistaphylococcal

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activity of lysostaphin in vitro more than 1,000-fold.⁶ Although lysostaphin demonstrated potent efficacy against MRSA infections in different animal models,^{10–12} its therapeutic potential was hampered by the emergence of bacterial resistance.^{10,13} In light of our previous results, showing enhancement of the antimicrobial effectiveness of lysostaphin against MRSA when combined with AMPs in vitro,⁶ we moved forward with an in vivo experiment in a mouse model of MRSA skin infection.

Materials and methods

Bacterial isolate

We obtained the community-acquired MRSA strain NRS384 (MRSA USA 300-0114) isolated from a wound from a patient in Mississippi, USA. The strain is resistant to erythromycin and tetracycline; positive for *mec* (subtype IV); *pvl*^{+/+}; multilocus sequence type 8⁻; eGenomic *spa* type 1, eGenomic *spa* repeats YHGFMBQBLO; Ridom *spa* type t008; *agr* grp I.

Peptides and antibacterial agents

RR and RRIKA were synthesized by GenScript (Piscataway, NJ, USA). Recombinant lysostaphin was purchased from Ambi Products (Lawrence, NY, USA). Linezolid and fusidic acid were purchased from Chem-Impex International (Wood Dale, IL, USA).

Mouse infection

Female BALB/c mice (6–8 weeks old) were obtained from Harlan Laboratories (Indianapolis, IN, USA). All procedures were approved by the Purdue University Animal Care and Use Committee (1207000676). The murine model of MRSA skin infection has been described before.^{14,15} Eight groups of mice (n=5) were inoculated with 40 μ L of MRSA USA 300 (3×10^7 CFU) intradermally. Forty-eight hours after infection and formation of an open wound, six groups were treated topically with either 2% fusidic acid, 2% RR, 2% RRIKA, 1% RRIKA, 0.5% lysostaphin, or 1% RRIKA plus 0.5% lysostaphin formulated in 20 mg petroleum jelly. One group received vehicle only (petroleum jelly), and the last group was treated orally with linezolid (25 mg/kg). All groups were treated twice a day for 3 days. Twenty-four hours after the last treatment, mice were humanely killed, the area around the wound lightly swabbed with 70% ethanol, and the wound (around 1 cm²) excised for bacterial counting after homogenization in 1 mL tryptic soy broth.

Cytokine detection

Enzyme-linked immunosorbent assay (ELISA) development kits for detection of cytokines were purchased from

R&D Systems (Minneapolis, MN, USA). Homogenized skin lesions after bacterial counting were centrifuged at 15,000 rpm for 10 minutes. The supernatants were removed and kept at -20°C until analyzed. The supernatants were examined for cytokine production: tumor-necrosis factor (TNF)- α , interleukin (IL)-6, and IL-1 β using ELISA as described before.¹⁶ Cytokine levels were expressed as percentage change relative to negative control.

Statistical analyses

Data are presented as means \pm standard deviation. Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA, USA). *P*-values were calculated by the two-tailed Student's *t*-test. *P*-values < 0.05 were considered significant.

Results and discussion

As shown in Figure 1, all treatments significantly reduced the mean bacterial counts compared with the control group ($P \leq 0.01$). Among groups treated with monotherapy, the group treated with 2% RRIKA had the highest reduction in CFU ($2.08 \pm 0.20 \log_{10}$), followed by 2% fusidic acid ($1.94 \pm 0.36 \log_{10}$), 2% RR ($1.83 \pm 0.30 \log_{10}$), and linezolid ($1.74 \pm 0.53 \log_{10}$). Groups treated with 0.5% lysostaphin or 1% RRIKA achieved a significant reduction of $1.79 \pm 0.56 \log_{10}$ and $1.08 \pm 0.36 \log_{10}$, respectively, when compared with

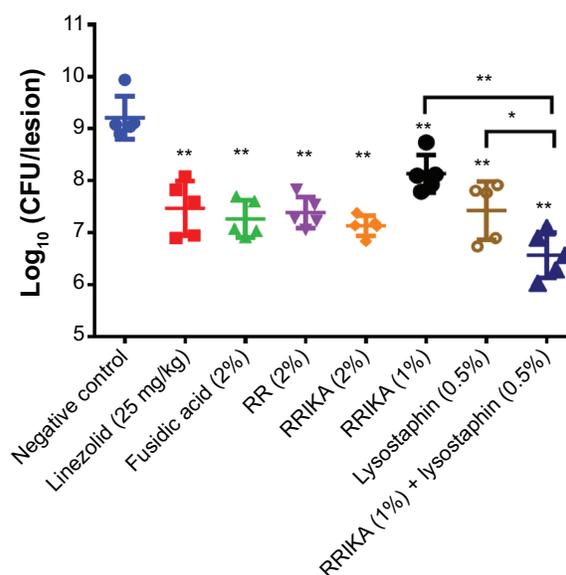


Figure 1 Efficacy of treatment of methicillin-resistant *Staphylococcus aureus* skin lesions with RRIKA (2%), RR (2%), fusidic acid (2%), linezolid (25 mg/kg), RRIKA (1%), lysostaphin (0.5%), combined therapy of RRIKA (1%) and lysostaphin (0.5%), and petroleum jelly (negative control) twice daily for 3 days. The two-tailed Student's *t*-test was used to determine statistical significance (a *P*-value < 0.05 was considered significant).

Notes: * $P < 0.05$; ** $P \leq 0.01$.

the control group ($P \leq 0.01$) (Figure 1). The combination therapy (0.5% lysostaphin with 1% RRIKA) was significantly more effective than treatment with RRIKA or lysostaphin alone, and achieved statistically significant bacterial reduction of $2.65 \pm 0.44 \log_{10}$ compared to the negative control ($P \leq 0.01$). The bacterial reduction in the combined therapy was also statistically significant compared to monotherapy

($P < 0.05$) or control antibiotics ($P < 0.05$). Previously, RR and RRIKA showed potent immunomodulatory activities in vitro through inhibition of mitogen-activated protein kinase (MK2).^{17,18} In this study, topical treatment with RRIKA and RR significantly reduced TNF α and IL-6 production in MRSA skin lesions. As shown in Figure 2A and B, 2% RR and 2% RRIKA reduced the TNF α level by 49% and

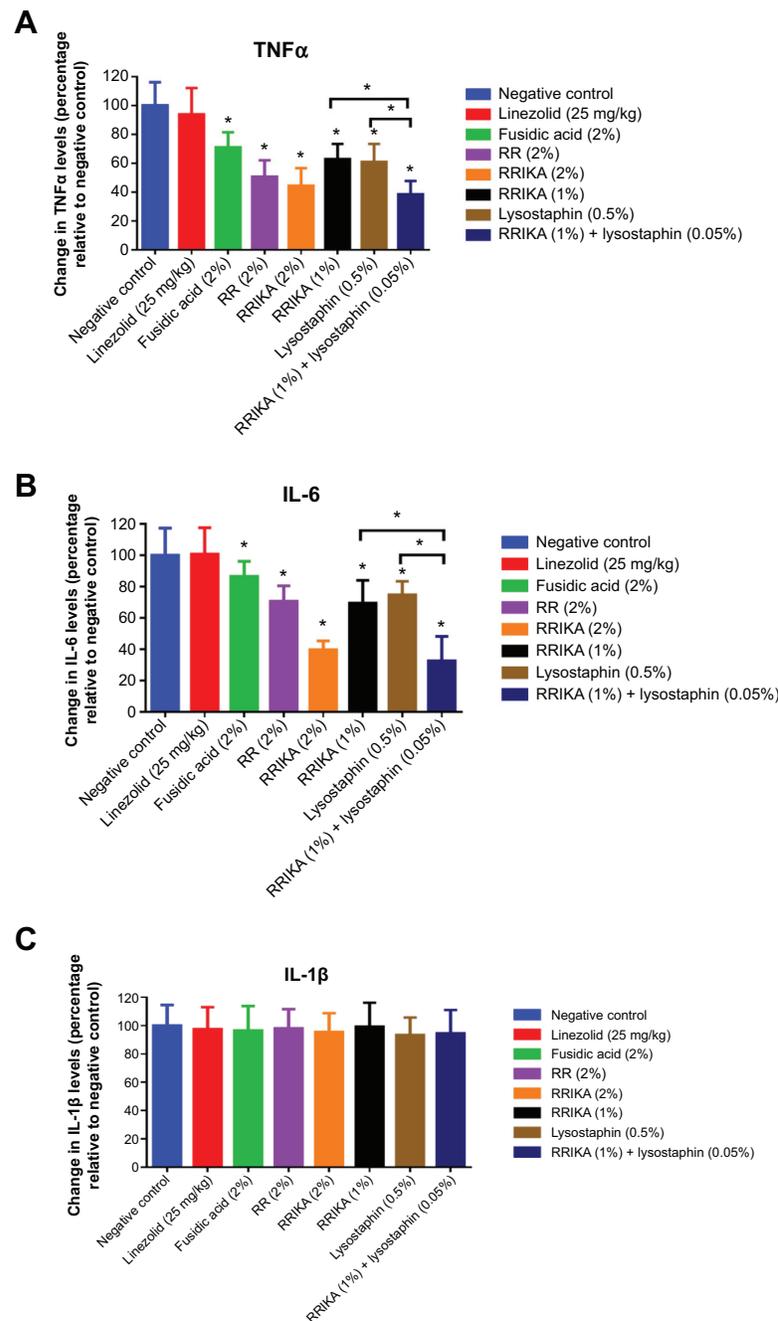


Figure 2 The effect of peptides on cytokines TNF α (A), IL-6 (B), and IL-1 β (C) production in methicillin-resistant *Staphylococcus aureus* skin lesions. Tissue homogenate supernatants were examined for cytokine production using the enzyme-linked immunosorbent assay. Cytokine levels were expressed as percentage change relative to negative control. Data are presented as means \pm standard deviation from duplicates consisting of four mice per group. Statistical analysis was calculated by the two-tailed Student's *t*-test. *P*-values < 0.05 were considered significant (*).

Abbreviations: TNF, tumor necrosis factor; IL, interleukin.

56%, respectively, while the IL-6 level was reduced by 29% and 60%, respectively. Treatment with 0.5% lysostaphin and 2% fusidic acid caused 39% and 29% reduction of the TNF α level, respectively, and 13% and 25% reduction of the IL-6 level, respectively, which is in agreement with previous findings.^{19,20} There was a synergistic anti-inflammatory response observed when 0.5% lysostaphin was combined with 1% RRIKA. The combined therapy significantly reduced TNF α and IL-6 levels by 62% and 67%, respectively. On the other hand, none of the treatments interfered with IL-1 β production, which is necessary for *S. aureus* clearance in cutaneous infections (Figure 2C).^{21–22}

In this study, topical application of RR and RRIKA was shown to be very effective in reducing the bacterial load in MRSA skin lesions. Moreover, peptides reduced the release of TNF α and IL-6, which might benefit the healing of infected wounds.^{23–27} In addition, the combination of RRIKA with lysostaphin was significantly more effective in the treatment of MRSA skin lesions than treatment with either peptide alone. This combination therapy is also expected to overcome some of the limitations associated with lysostaphin monotherapy through hindering the emergence of bacterial resistance and lowering the required therapeutic dose.

In conclusion, our findings in MRSA skin lesions should significantly impact and inform efforts to use a combination of anti-inflammatory and AMP therapies as novel topical treatment options for multidrug-resistant pathogens.

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Disclosure

The authors report no conflicts of interest in this work.

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