Lipopeptide Laur-CKK-NH₂ dimer preserves daptomycin susceptibility and enhances its activity against Enterococcus faecalis

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Objectives: An experimental study was performed to evaluate both in vitro and in vivo the kind of interaction between the Laur-CKK-NH₂ dimer and daptomycin using two Enterococcus faecalis strains with different patterns of susceptibilities.

Methods: We evaluated whether selection for daptomycin-resistant E. faecalis could be prevented in vitro by combining daptomycin with the Laur-CKK-NH₂ dimer. The strains were serially exposed in broth to 2-fold step-wise increasing concentrations of daptomycin alone or in combination with a fixed concentration (0.25 × MIC) of the Laur-CKK-NH₂ dimer. We also performed an in vitro synergy study. For in vivo studies, a mouse model of enterococcal sepsis was used.

Results: In vitro experiments: exposure to daptomycin alone gradually selected for enterococci with increased MICs; and the Laur-CKK-NH₂ dimer showed a positive interaction with daptomycin and was able to prevent the resistance. In vivo experiments: the main outcome measures were lethality and quantitative blood cultures; and the Laur-CKK-NH₂ dimer combined with daptomycin exhibited the highest efficacy for all main outcome measurements.

Conclusions: These results highlight the potential usefulness of combining daptomycin with the Laur-CKK-NH₂ dimer. The combination provides a future therapeutic alternative for the treatment of enterococcal severe infections.

Keywords: enterococci, E. faecalis, sepsis

Introduction

Gram-positive bacteria are common nosocomial pathogens. 1 The vancomycin-resistant enterococci have spurred the development of newer antimicrobial agents active against vancomycin-resistant organisms. 2 Daptomycin is a fermentation product of Streptomyces roseosporus and the first lipopeptide developed for clinical use. 3,4 Daptomycin inserts in the outer leaflet of the bacterial membrane, inducing leakage of the cytosolic content and a rapid bactericidal effect. As a result of this unique mechanism of action, it possesses a relatively prolonged concentration-dependent post-antibiotic effect in vitro. No cross-resistance has been observed with any other class of antibiotic. 5

Antimicrobial peptides are widely distributed in nature as a part of the innate immune system in multicellular organisms to counteract the action of microbes. 6 It has been shown that conjugation of aliphatic fatty acids to antimicrobial peptides results in an increase in their antibacterial and antifungal activities. 7 Lauric acid is an aliphatic monocarboxylic acid that has 12 carbons. In this study, a new compound [Laur-Cys-Lys-Lys-NH₂ (Laur-CKK-NH₂) dimer] was synthesized by conjuction of this acid with the amino acids cysteine-lysine-lysine. Reports have shown synergistic effects when antimicrobial peptides are combined with some clinically used antibiotics. 7 However, the ability of partner drugs to interfere with the development of antibiotic resistance was poorly investigated.
In order to broaden this knowledge, we explored both in vitro and in vivo the kind of interaction between the Laur-CKK-NH₂ dimer and daptomycin using two Enterococcus faecalis strains with different patterns of susceptibilities.

Materials and methods
Vancomycin-susceptible E. faecalis ATCC 29212 and vancomycin-resistant E. faecalis ATCC 51299 were used. Daptomycin was provided by Novartis Italia S.p.A (Varese, Italy). Laur-CKK-NH₂ was synthesized manually using solid-phase methodology on polystyrene AM-RAM resin using 9-fluorenlymethoxy carbonyl (Fmoc) chemistry. ⁸

MICs were determined in cation-adjusted Mueller–Hinton (MH) broth II (50 mg/L Ca²⁺) using a broth microdilution method according to the CLSI (formerly NCCLS) guidelines. ⁹ Experiments were performed in triplicate.

To evaluate progressive reduction in susceptibility to daptomycin, a series of tubes containing 2-fold increasing concentrations of daptomycin were inoculated with 10⁷ cfu/mL. Following 24 h of incubation at 35°C, 0.1 mL samples from the tubes containing the highest antibiotic concentration and still showing turbidity were used to inoculate a new series of tubes containing antibiotic dilutions, and so on for a total of seven consecutive passages. To examine the potential for the Laur-CKK-NH₂ dimer to preserve daptomycin susceptibility, the experiments described above were repeated in the presence of 0.25× MIC of the lipopeptide. The MIC of daptomycin was recorded after each passage. Each experiment was performed in triplicate. The stability of changes in susceptibility to daptomycin was assessed by serial passage of the organisms on antibiotic-free medium for 5 consecutive days.

For killing assays, samples of exponentially growing bacteria were resuspended in the presence and absence of human albumin (40 mg/mL) in fresh Ca²⁺-supplemented MH broth II at approximately 10⁷ cells/mL and exposed to each agent (final concentrations 1×, 2× or 4× MIC) for 0, 0.5, 1, 2, 4, 6, 12, 18 and 24 h at 37°C. After these times, samples were serially diluted and plated onto MH agar plates to obtain viable colonies.

Interaction studies were performed by the checkerboard titration method using 96-well polypropylene microtitre plates. The plates of drug dilutions used were 0.125–64 mg/L for daptomycin, and 0.25–256 mg/L for both strains. The FIC indices were interpreted as follows: <0.5, synergy; 0.5–4.0, indifferent; and >4.0, antagonism. ¹⁰

For the cytotoxicity assay, A-549 cells from human lung carcinoma (BioWhittaker Inc., Walkersville, MD, USA) were cultured in 25 cm² tissue culture flasks. The medium consisted of Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal calf serum (Bio-Whittaker). The cytotoxicity of the Laur-CKK-NH₂ dimer at 1× or 2× MIC was determined by the CellTiter 96 AQ cell proliferation assay (Promega Corp., Lyon, France).

BALB/c male mice weighing 25 to 35 g were used for all in vivo experiments. The study was approved by the animal research ethics committee of the I.N.R.C.A.–I.R.R.C.S., Polytechnic University of Marche, Ancona, Italy.

Exponentially growing bacteria were resuspended in brain heart infusion broth and then centrifuged at 1000 g for 15 min. The supernatant was discarded and the bacteria were resuspended in sterile saline to achieve a concentration of approximately 1×10⁸ cfu/mL. All animals were anaesthetized by intramuscular injection of ketamine (30 mg/kg). Mice were injected intraperitoneally with 0.2 mL of the bacterial suspensions: (i) 2.0×10⁷ cfu of E. faecalis ATCC 29212; or (ii) 2.0×10⁷ cfu of E. faecalis ATCC 51299. Immediately after bacterial challenge, the mice were randomized to receive intraperitoneally isonicotinic acid hydrazide solution (control group), 6 mg/kg daptomycin, 1 mg/kg Laur-CKK-NH₂ dimer and finally 1 mg/kg Laur-CKK-NH₂ dimer plus 6 mg/kg daptomycin in a volume of 0.2 mL per dose. Each group included 15 mice. The animals were returned to individual cages and monitored for the subsequent 72 h. The endpoints were lethality rates and quantitative blood cultures. Toxicity was evaluated on the basis of the presence of drug-related adverse effects (local signs of inflammation, weight loss, vomiting, diarrhoea and fever) in a supplementary lipopeptide-treated group without challenge.

Blood samples for culture were obtained from the tail vein by aseptic percutaneous puncture 24 h after bacterial challenge. The animals that died before this time were not tested. To perform quantitative bacterial cultures, blood samples were serially diluted and a 0.1 mL volume of each dilution was spread on blood agar plates and cultured at 35°C for 48 h and the cfu counted. The limit of detection was <10 cfu/mL.

For statistical analysis, lethality rates between groups were compared by Fisher’s exact test. Data from quantitative blood cultures are presented as means ± SD; comparisons between groups were made by analysis of variance. Post hoc comparisons were performed by Bonferroni’s test. Significance was accepted when the P value was ≤0.05.

Results and discussion
Daptomycin exhibited MICs of 1 and 4 mg/L for E. faecalis ATCC 29212 and E. faecalis ATCC 51299 strains, respectively. The Laur-CKK-NH₂ dimer showed MICs of 8 mg/L for both strains.

Combination studies demonstrated synergy between the Laur-CKK-NH₂ dimer and daptomycin; FIC indices ranged between 0.312 and 0.458. A cytotoxic effect of the Laur-CKK-NH₂ dimer was practically absent at the concentrations tested; percentage cytotoxicity = 3.2%.

Daptomycin and the Laur-CKK-NH₂ dimer antibacterial profiles differed when testing 4× MIC and 1× or 2× MIC. Both agents at 4× MIC were rapidly bactericidal (≤6 h) with >5 log₁₀ reduction in the initial inocula for the two enterococcal strains, regardless of the presence of albumin. Daptomycin at 1× or 2× MIC exhibited similar final colony counts at 0 h and 24 h regardless of the presence of albumin, with >3 log₁₀ cfu/mL reduction at 6 h for both strains. Lower activity was demonstrated by the Laur-CKK-NH₂ dimer at 1× or 2× MIC with an approximately 2 log₁₀ cfu/mL reduction at 6 h for both enterococcal strains.

Serial exposure to daptomycin alone resulted in a reduction in susceptibility. In contrast, addition of 0.25× MIC of the Laur-CKK-NH₂ dimer preserved susceptibility to daptomycin (Figure 1).

As shown in Table 1, when mice were challenged with both strains and treated with saline (control group), the rate of lethality was 100% within 72 h. In contrast, treatment with drugs demonstrated significantly higher efficacy (P<0.05). For E. faecalis ATCC 29212, lethality rates of 26.7% and 46.7% were observed for the groups treated with daptomycin and the Laur-CKK-NH₂ dimer, respectively. For E. faecalis ATCC 51299, the lethality rate was 33.3% for daptomycin and 46.7% for the Laur-CKK-NH₂ dimer. The combination of daptomycin and the Laur-CKK-NH₂ dimer showed significantly lower lethality rates (6.7%) for both of the strains. Quantitative blood cultures showed high bacterial numbers in control groups (Table 1). The Laur-CKK-NH₂ dimer demonstrated good antibacterial activity against the two strains, with an approximately 2 log₁₀ cfu/mL reduction in bacterial growth. Daptomycin alone showed higher activity against the two strains, with an approximately 4 log₁₀ cfu/mL reduction compared with controls. When daptomycin was combined with the lipopeptide, the positive interaction produced the lowest bacterial counts (6 log₁₀ cfu/mL reduction compared with controls). The group treated with the combination had significantly lower bacterial counts compared
Lipopeptide and daptomycin combination for *E. faecalis* infection

Figure 1. Selection of *E. faecalis* ATCC 29212 and *E. faecalis* ATCC 51299 daptomycin-resistant mutants after seven passages of exposure to daptomycin alone or combined with Laur-CKK-NH$_2$ (0.25× MIC). Filled squares and triangles, bacteria exposed to daptomycin alone; open squares and triangles, bacteria exposed to daptomycin plus Laur-CKK-NH$_2$.

Table 1. Effect of daptomycin and Laur-CKK-NH$_2$ in a mouse model of *E. faecalis*-induced sepsis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Lethality$^a$ [dead/total (%)]</th>
<th>Bacterial counts in blood (cfu/mL)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>E. faecalis</em> ATCC 29212</td>
<td><em>E. faecalis</em> ATCC 51299</td>
</tr>
<tr>
<td>No treatment (control group)</td>
<td>15/15 (100)</td>
<td>15/15 (100)</td>
</tr>
<tr>
<td>Daptomycin 6 mg/kg</td>
<td>4/15 (26.7)$^{c,d}$</td>
<td>5/15 (33.3)$^{c,d}$</td>
</tr>
<tr>
<td>Laur-CKK-NH$_2$ 1 mg/kg</td>
<td>7/15 (46.7)$^c$</td>
<td>7/15 (46.7)$^c$</td>
</tr>
<tr>
<td>Daptomycin 6 mg/kg plus Laur-CKK-NH$_2$ 1 mg/kg</td>
<td>1/15 (6.7)$^{d,e,o}$</td>
<td>1/15 (6.7)$^{d,e,o}$</td>
</tr>
</tbody>
</table>

$^a$Lethality was monitored for 72 h after challenge.
$^b$Mean±SD.
$^c$$P$≤0.05 (Fisher’s test) or $P$≤0.05 (Bonferroni’s test) versus the control group.
$^d$$P$≤0.05 (Fisher’s test) or $P$≤0.05 (Bonferroni’s test) versus the lipopeptide-treated group.
$^e$$P$≤0.05 (Fisher’s test) or $P$≤0.05 (Bonferroni’s test) versus the singly treated groups.

with the singly treated groups ($P$≤0.05). Finally, none of the animals had clinical evidence of drug-related adverse effects or hypersensitivity reactions. No changes in physiological parameters were observed in the supplementary lipopeptide-treated group without infection.

In the present study, we explored the interaction between the Laur-CKK-NH$_2$ dimer and daptomycin using two *E. faecalis* strains with different patterns of susceptibilities, first *in vitro* and then *in vivo*. In *in vitro* studies, we observed that the Laur-CKK-NH$_2$ dimer prevented the emergence of daptomycin resistance. The *in vitro* studies showed the presence of a synergistic effect between the Laur-CKK-NH$_2$ dimer and daptomycin. Nevertheless, the mechanism of this positive interaction remains largely unknown; a possible role of lipopeptides in such synergy is the disruption of cell wall components, allowing enhanced penetration and activity of daptomycin. The synergistic pattern clearly found in *in vitro* studies was also observed in an *in vivo* setting. Indeed, the best results for mortality rates and bacteraemia were obtained when the Laur-CKK-NH$_2$ dimer was combined with daptomycin, suggesting that their mode of action might be complementary. In conclusion, our study showed the ability of the Laur-CKK-NH$_2$ dimer to be synergistic and to prevent daptomycin resistance, suggesting a potential therapeutic option for severe enterococcal infections.

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Transparency declarations
None to declare.

References