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Synthetic epidermicin NI01 can protect *Galleria mellonella* larvae from infection with *Staphylococcus aureus*

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Objectives: Epidermicin is a novel antimicrobial peptide that has potent activity against Gram-positive pathogens, including methicillin-resistant *Staphylococcus aureus*, and it may have potential for use in therapy for infections caused by these bacteria, though *in vivo* efficacy needs to be demonstrated. *Galleria mellonella* larvae have recently been introduced as an alternative *in vivo* model to mammalian systems and here we examined the ability of a synthetic version of epidermicin to protect *G. mellonella* larvae from infection with *S. aureus* strains.

Methods: *G. mellonella* larvae were infected with $\sim 2.5 \times 10^6$ cells per larva and then treated with vancomycin or epidermicin and survival recorded over a 120 h period. Vancomycin was used at up to 50 mg/kg and epidermicin at up to 200 mg/kg with administration of treatments occurring 0–2 h post-infection.

Results: Epidermicin was shown to be non-toxic and did not stimulate the *G. mellonella* immune system. When administered 2 h post-infection at a maximum dose of 200 mg/kg, epidermicin significantly increased survival in larvae; however, altering the dosage regimen by reducing the time to administration to 30 min post-infection increased the potency of the peptide.

Conclusions: This is the first report of antimicrobial activity of an artificially synthesized peptide from the type IIc bacteriocin family. The novel peptide protects *G. mellonella* larvae from infection with both methicillin-susceptible and -resistant *S. aureus*, although the pharmacodynamic properties are not yet optimal.

Keywords: antimicrobial peptides, bacteriocins, vancomycin, methicillin-resistant *Staphylococcus aureus*

Introduction

Currently there is an urgent need to identify and develop novel antibiotics to combat infections caused by drug-resistant bacterial pathogens. Epidermicin NI01, an unmodified bacteriocin produced by *Staphylococcus epidermidis* strain 224, exhibits potent antimicrobial activity towards a wide range of pathogenic Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci and biofilm-forming *S. epidermidis* strains.[1] Epidermicin is a member of a recently described family of type IIc bacteriocins. The activity of synthetic peptides from this family has not been investigated.

The recent introduction of *Galleria mellonella* larvae as a model host provides an alternative *in vivo* model to mammalian systems. It fulfils many of the basic requirements of a useful animal model, most notably the immune system shares a high degree of structural and functional similarity with that of mammals[2] and involves both cellular and humoral defences. The humoral response consists of melanization, haemolymph clotting and production of antimicrobial peptides, whereas the cellular responses consist of phagocytosis, nodulization and encapsulation. In addition, *G. mellonella* has a short life cycle and the larvae are easy to breed, inexpensive and widely available. Tests using larvae as an *in vivo* model are considered simple to initiate, predictable, reproducible and quantifiable, making it a desirable first line of *in vivo* testing.[3] Larvae of *G. mellonella* have been used previously as a model host for studying the virulence of pathogenic microbes, including *S. aureus*,[4] *Streptococcus* species,[5,6] *P. aeruginosa*,[7] *Bacillus cereus*,[8] dermatophytes,[9] *Aspergillus* species,[10] *Cryptococcus neoformans* and *Candida albicans*.[11] The larvae have also been used to study the efficacy of various antimicrobial agents.[4]

The main objective of this study was to evaluate the antimicrobial activity of a synthetic epidermicin NI01 peptide against *S. aureus* using the wax moth larva *G. mellonella* as a model host and to compare the activity with the effect of vancomycin.

Materials and methods

Bacterial strain

Methicillin-susceptible *S. aureus* (MSSA) strain ATCC 11195 and a clinical isolate of MRSA were employed during the course of this study. The MRSA strain had been previously collected by staff at a hospital bacteriology laboratory in Manchester, UK, and is part of a local culture collection. The strains were routinely cultured in LB broth (10 g/L tryptone and 5 g/L yeast extract, with no salt) or Columbia Blood Agar (CBA; Oxoid, UK) incubated at 37°C.

Insect larvae

Sixth instar larvae of *G. mellonella* were obtained from Live Foods Ltd (Rooks Bridge, UK). Larvae were stored in the dark at 4°C and used within 2 weeks of receipt.

A Hamilton syringe (26S gauge, 50 μL capacity) was used to inoculate larvae with *S. aureus* and for introduction of treatments or control solutions into the larvae. The methods of Desbois and Coote[4] were followed.

Antimicrobial agents

Epidermicin was obtained from Almac Sciences (East Lothian, UK). As the native peptide is N-formylated, and such modifications are conserved in bacteriocins, the peptide used in this study was synthesized with this modification. As the peptide adheres to polystyrene 96-well microtitre plates, the peptide was dissolved in peptide suspension buffer (0.01% acetic acid buffer containing 0.2% BSA) and kept at −20°C until required for use. Vancomycin hydrochloride was obtained from Sigma-Aldrich Ltd (Poole, UK) and was prepared using sterile deionized water.

The MICs of epidermicin and vancomycin were determined by a modified 2-fold microtitre broth dilution method as described by Wu and Hancock.[12]

Assessment of the toxicity of epidermicin

The toxicity of epidermicin was determined using repeated doses of 200 mg/kg into the larvae (which exceeded the MIC required for strain ATCC 11195) at 0, 24 and 48 h. Two groups of 10 larvae were inoculated in the last left proleg. Three negative control groups (10 larvae each) were used in this experiment: one group that underwent no manipulation whatsoever, one group injected with PBS only (10 μL) and one group injected with peptide suspension buffer only (10 μL). Test and control larvae were stored in sterile Petri dishes in the dark at 37°C for 120 h. Larvae were inspected every 24 h and were considered dead if they did not move in response to touch. The endpoint of the experiment was the death of all the larvae in the experimental groups or the transition of larvae into pupae, or the end of the experiment time (120 h).

Effect of epidermicin on haemocyte densities in *G. mellonella*

Groups of six larvae were injected with peptide suspension buffer (control) and another group with epidermicin at 10 mg/kg and were kept at 37°C. Haemocyte density was determined after 4 and 24 h. The haemolymph of three larvae per treatment was collected in a pre-chilled 1 mL tube containing a few granules of thiourea. Haemolymph was then diluted 1 in 10 in cold PBS containing 0.37% (v/v) mercaptoethanol (Sigma-Aldrich). Haemocytes were counted on a haemocytometer and their density in haemolymph was calculated. No attempt was made to discriminate between the different haemocyte subtypes.

Measuring bioavailability of epidermicin

Three groups of 30 larvae were injected with epidermicin at doses of 2, 4 and 20 μg/larvae (equivalent to 10, 20 and 100 mg/kg, respectively) and kept at 37°C. Three larvae of each group after 5, 10, 15, 20, 30, 40, 50, 60, 90 and 120 min were immersed in 70% alcohol and dried on sterile filter paper before removing a thoracic leg of the larva using a sterile blade and the haemolymph was collected in a pre-chilled 1 mL tube containing a few grains of thiourea (Sigma-Aldrich) to prevent melanization. Three 2-fold dilutions of the haemolymph were prepared and screening of the peptide activity was carried out using an agar well diffusion method[13] and *S. aureus* strain ATCC 11195 as indicator.

Survival assay

S. aureus strain ATCC 11195 was grown in LB at 37°C for 18 h and the optical density at 600 nm (OD_{600}) measured. The bacterial cells were then collected and washed twice before being diluted with PBS so that 10 μL of bacterial suspension contained 3×10^5–5×10^6 cfu (using previously established cfu/mL counts). One group of 10 larvae per concentration were inoculated in the last left proleg. Two negative control groups (10 larvae each) were used in this and all following experiments: one group underwent no manipulation, while the other group was injected with PBS only. Test and control larvae were stored in Petri dishes in the dark at 37°C for 120 h and survival monitored as described above.

Protection from infection using vancomycin or epidermicin

Cells of *S. aureus* strains ATCC 11195 and the MRSA clinical isolate were grown as described above before being washed twice using PBS. The OD_{600} of the bacterial suspension was adjusted to 0.45 to give 2.5×10^8 cfu/mL and larvae injected in the last left proleg with 10 μL (2.5×10^6/larva).

The viability of all larvae was confirmed 2 h post-inoculation. Then the first treatment doses of vancomycin (1, 10 and 50 mg/kg), epidermicin (1, 10, 100 and 200 mg/kg) or PBS (control) were administered. Test and control larvae were stored in Petri dishes in the dark at 37°C for 120 h. Repeat treatment doses of vancomycin, epidermicin or PBS were given at 24 and 48 h. Larvae were inspected every 24 h for 120 h.

Following initial experiments, the antistaphylococcal activity of epidermicin was determined using a single dose of 1, 10 or 100 mg/kg administered 0, 15, 30 or 60 min after inoculation with strain ATCC 11195.

Statistics

Survival analysis and statistical significance were determined using the log-rank test and the Kaplan–Meier survival curves were plotted using SPSS v.19.

Results and discussion

In the present study, *G. mellonella* wax moth larvae were used as a non-mammalian model system to evaluate the activity of the novel antistaphylococcal peptide epidermicin. The activity of bacteriocins has not previously been tested in this manner in *G. mellonella*. The *G. mellonella* model has been cited as a suitable pre-screen for efficacy assessment of antistaphylococcal agents.[3]

Epidermicin NI01 is not toxic towards *G. mellonella* larvae and has no effect on haemocyte densities

The MICs of epidermicin and vancomycin for *S. aureus* ATCC 11195 were determined to be 0.156 mg/L and 0.25 mg/L, respectively.

The MIC of synthetic epidermicin is lower than we have previously reported for natively synthesized peptide. Peptide suspension buffer or epidermicin peptide administered at concentrations up to 40 μg/larvae (200 mg/kg) did not result in death or visible injury, indicating that the peptide was not toxic towards the larvae. This supports our previous work,[1] where we demonstrated that epidermicin is not toxic towards murine fibroblasts at concentrations of up to 100 times the MIC and indicates that epidermicin may have limited toxicity in mammalian systems.[3,4]

To investigate whether administration of epidermicin could evoke an antimicrobial immune response independent of its antimicrobial properties, the ability of epidermicin to alter the haemocyte density was determined, since this is an indication of larval susceptibility to infection. The peptide does not boost this particular element of the larval immune system, as no significant differences were observed between haemocyte density in the peptide-treated (cell count of 296 797 ± 6292 at 24 h) and control (304 237 ± 19 105) groups ($P = 0.55–0.98$). It is possible that the rate of phagocytosis may be increased by the peptide, but we are confident that any efficacy against *S. aureus* is not a manifestation of a simulation of *G. mellonella* antimicrobial peptides.

Epidermicin has appreciable bioavailability in G. mellonella larvae

To determine the bioavailability of the epidermicin after treating the larvae, a bioactivity assay was performed using different concentrations of the peptide and different haemolymph collection times. The haemolymph retained antistaphylococcal activity only at an epidermicin dosage of 100 mg/kg and only if assayed at <50 min post-administration (data not shown). This suggests that the peptide is either being degraded or activity is being limited by complex formation with constituents of the haemolymph. The peptide is highly stable[1] *in vitro* and further investigations are warranted to improve the bioavailability of epidermicin in this model.

Epidermicin can protect G. mellonella larvae from infection with MSSA and MRSA

The effect of *S. aureus* ATCC 11195 on the survival of the larvae appeared to be dose dependent, where survival was reduced with increasing inoculum of *S. aureus*. Although all inoculum doses showed a reduction in larval survival, inoculum doses ranging between 1.25×10^6 and 2.5×10^6 were selected for use in further studies, as they showed significant differences in larval survival compared with the uninfected control group and a gradual reduction in survival rate covering the whole experiment period (Figure 1). Vancomycin, even at a low concentration of 1 mg/kg, showed a significant ($P = 0.001$) effect on larval survival compared with the untreated control group (not shown).

In initial experiments, the highest concentration (200 mg/kg) of epidermicin (administered 2 h post-infection) significantly increased the survival of larvae infected with either MSSA strain ATCC 11195 or the clinical MRSA strain compared with untreated controls (Figures 2 and 3). The lower concentration (100 mg/kg), which correlated to 100 times the MIC for strain ATCC 11195, failed to achieve statistical significance under the standard dosage regimen. The bioavailability assay indicated that

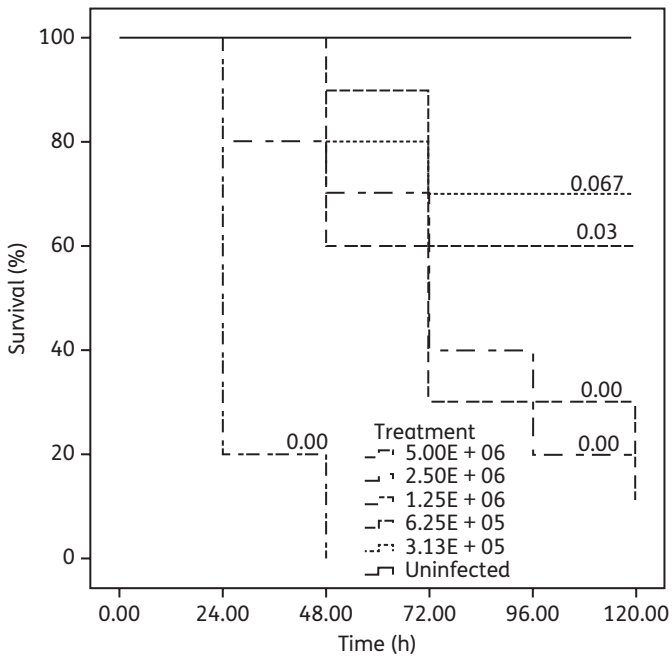

Figure 1. Survival of *G. mellonella* larvae infected with different concentrations of *S. aureus* strain ATCC 1195. Numbers in the graph represent the *P* value of each treatment compared with the uninfected control.

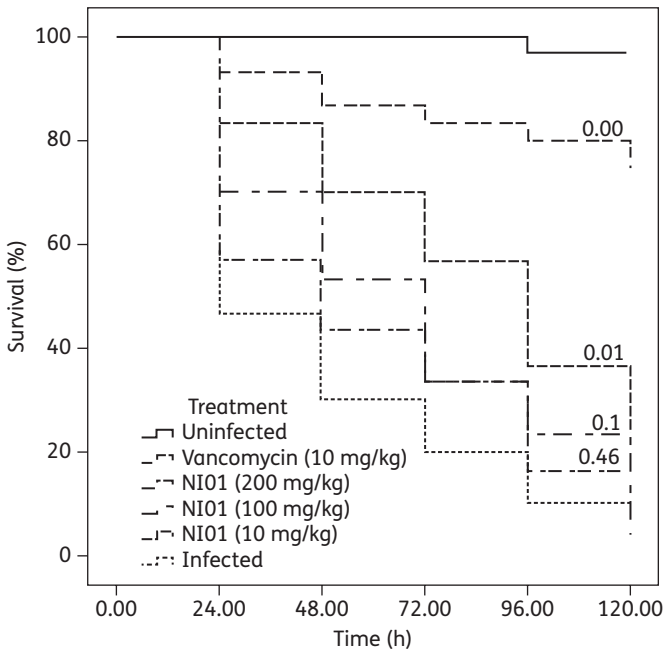

Figure 2. Survival of *G. mellonella* larvae infected with *S. aureus* strain ATCC 1195 and treated with various doses of epidermicin 2 h post-infection. Numbers in the graph represent the *P* value of each treatment compared with the untreated, infected control.

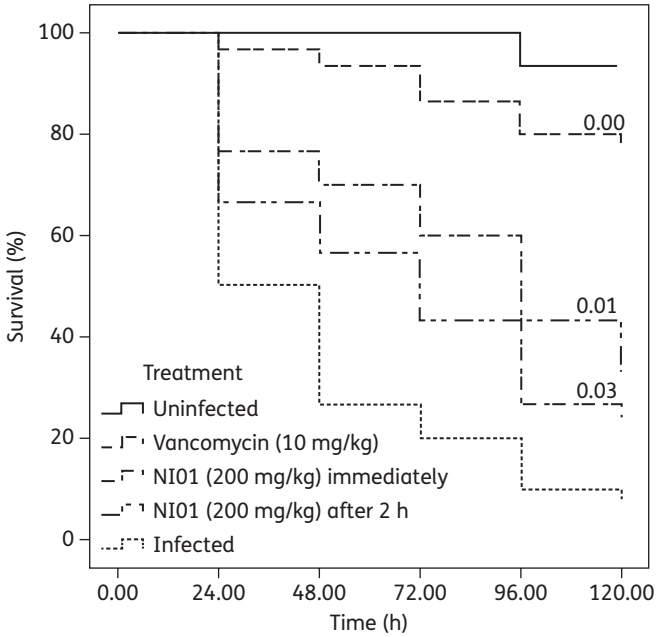

Figure 3. Survival of *G. mellonella* larvae infected with a clinical MRSA strain and treated with 200 mg/kg epidermicin at various times post-infection. Numbers in the graph represent the *P* value of each treatment compared with the untreated, infected control.

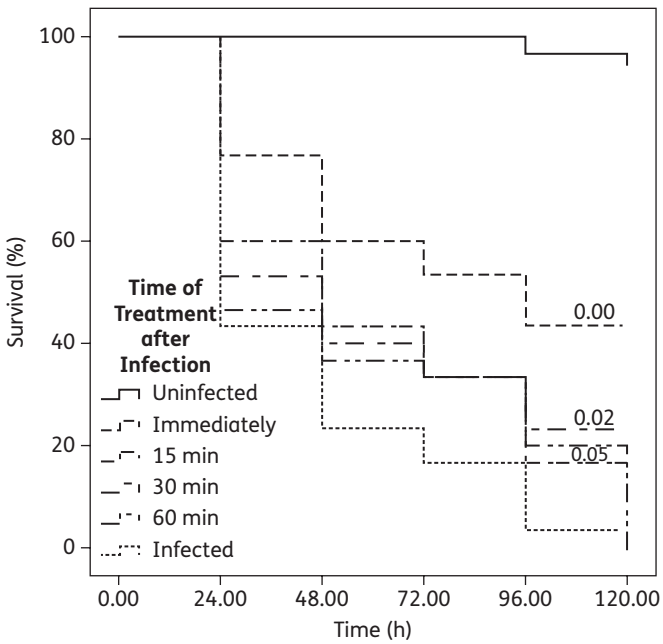

Figure 4. Survival of *G. mellonella* larvae infected with *S. aureus* strain ATCC 1195 and treated with 100 mg/kg epidermicin at various times post-infection. Numbers in the graph represent the *P* value of each treatment compared with the untreated, infected control.

epidermicin activity was only detectable up to 50 min post-administration. More sensitive detection assays such as mass spectrophotometric analysis of the haemolymph may confirm if epidermicin is being degraded in the larvae.

In all assays, comparability to the control treatment of vancomycin was not achieved. We do not consider this to be a significant limitation, as vancomycin is a very effective antistaphylococcal agent with proven efficacy in this model.[4,14] In addition, this work did not aim to demonstrate superiority over the current standard treatment; we merely used vancomycin as a positive control agent.

Numerous other factors could be responsible for the lack of pronounced activity following therapy with epidermicin, including the virulence of the strains used or the *in vivo* distribution and bioavailability of the peptide. To further examine the antibacterial activity of the peptide, a modified dosing regimen was developed using a single dose of peptide at 100 mg/kg at different times post-infection with MSSA strain ATCC 11195. This modified protocol showed a significant association between time of treatment and survival of the larvae, and treatments up to 30 min post-infection significantly increased larval survival (Figure 4). Further investigations are required to examine what is underlying the reduced efficacy of epidermicin administered outside the 30 min window. Although the exact peptide–host interaction is not clear, our data suggest that epidermicin has some pharmacokinetic limitations that need to be identified and eliminated in order to improve its effectiveness.

Conclusions

In conclusion, epidermicin does have *in vivo* antistaphylococcal efficacy and significantly improved the survival of *G. mellonella* larvae infected with representative strains of MRSA and MSSA. While this result is promising, it will be important to explore different dosages and to determine whether similar results can be obtained for other organisms, as epidermicin has potent activity against a range of Gram-positive pathogens. Increasing the bioavailability of the peptide is clearly also required. This is the first demonstration of the *in vivo* efficacy of a peptide from the type IIc group of unmodified bacteriocins, and the findings justify further development of epidermicin towards potential therapeutic use. Use of a synthetic peptide will expand the possibilities for rational development of analogues with altered activity.

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Transparency declarations

None to declare.

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