Mersacidin eradicates methicillin-resistant *Staphylococcus aureus* (MRSA) in a mouse rhinitis model

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**Objectives:** Methicillin-resistant *Staphylococcus aureus* (MRSA) often colonize the anterior nares, and nasal carriage remains the main source of bacterial dissemination. The aim of this study was to assess the *in vivo* activity of the lantibiotic mersacidin against MRSA colonizing nasal epithelia.

**Methods:** The efficiency of mersacidin in the eradication of MRSA was tested employing mice pretreated with hydrocortisone and inoculated intranasally either three or six times with a bacterial suspension.

**Results:** In mersacidin-treated animals, pre-colonized with MRSA, bacteria could not be detected in blood, lungs, liver, kidney, spleen or nasal scrapings and there were no lesions manifested after intraperitoneal drug application. Blood samples from infected mice obtained 2 h after mersacidin therapy revealed anti-MRSA activity in a serum bactericidal test. Moreover, elevated interleukin-1β and tumour necrosis factor-α titres were noticed in the pre-infected but not in cured animals. In contrast, mersacidin did not induce differences in the cytokine profiles of treated uninfected control mice.

**Conclusions:** In the mouse rhinitis model, mersacidin was able to eradicate MRSA colonization. The site of action (epithelium versus blood) of mersacidin needs to be further explored.

Keywords: lantibiotics, mouse model, MRSA nasal carriage

**Introduction**

*Staphylococcus aureus* remains one of the most intensively investigated bacterial species. As a human and animal pathogen, it can cause a variety of nosocomial and community-acquired infections ranging from minor skin abscesses to serious, potentially life-threatening diseases, such as bone and soft tissue intra-surgical infections, sepsis and invasive endocarditis.1,2 In terms of resistance, *S. aureus* infections pose an ever increasing problem. Methicillin-resistant *S. aureus* (MRSA) have spread worldwide, with infection rates that are <5% in the Scandinavian countries but >40% in Japan, the USA and southern Europe,1 and the first vancomycin-resistant strains have been isolated in the USA.3,4 *S. aureus* can be carried asymptomatically on the nasal epithelium in healthy carriers; however, epidemiological studies link the carriage of *S. aureus* with a significantly higher risk for the development of staphylococcal diseases.5–8 Thus, the need to introduce effective bactericidal substances for treatment of nasal carriage and therapy of MRSA infections is pressing.

On several occasions it has been shown that mersacidin, a lantibiotic (lanthionine containing antibiotic), which is ribosomally produced by *Bacillus* sp. HIL Y-85, 54728 (Figure 1)9–11 can successfully inhibit growth of MRSA and other Gram-positive bacteria *in vitro*.12–14 Mersacidin inhibits bacterial cell wall biosynthesis by complexing lipid II.15–17 It is a 20 amino acid peptide, contains four ring forming amino acids and is characterized by chemical stability and a compact globular structure.18,19 Under *in vitro* conditions, the composition of the growth medium, time and co-culture conditions (e.g. anaerobic) of the indicator strains have minimal effects on the MICs and MBCs of mersacidin.20 Moreover, it has been shown that subcutaneously administered mersacidin cures systemic MRSA infections in mice and abscesses in rats.21

The aim of the present study was to assess *in vivo* the potential of mersacidin for curing nasal carriage of MRSA. In order to
Mersacidin eradicates MRSA

**Materials and methods**

**Bacterial strain**

The MRSA strain *S. aureus* 99308 (Lund University, Dept. Medical Microbiology, Dermatology, and Infection) was used in this study. It was identified according to standard laboratory procedures as *S. aureus* \(^{23}\) (including isolation on agar and tests for production of coagulase, clumping factor, coeitin and acid aerobically from trehalose and maltose). Resistance to methicillin was confirmed by growth on Mueller–Hinton agar (Oxoid Ltd, London, UK) with the addition of oxacillin (Sigma, St Louis, MO, USA) at a concentration of 6 mg/L with 4% NaCl (OXA-medium) after 18 h incubation at 30°C.

In order to adapt this human clinical isolate to optimal colonisation of the mouse nasal epithelium, the production of virulence factors by the bacteria was promoted, both in *vitro* and *in vivo*. Specifically, attempts were made to stimulate the production of a polysaccharide capsule by culture on Columbia agar (Becton Dickinson) with 5% sheep blood supplemented with 2% NaCl. Subsequently, in order to adapt the strain to the animal model, bacteria were passed through four healthy BALB/cA mice.

To this end, the bacteria were injected (200 μL) intraperitoneally with \(3 \times 10^6–10^8\) cfu. The following day, the animal was bled by heart puncture. Those staphylococci that could be isolated from liver samples were then passed through healthy mice by intranasal administration. The bacteria were reisolated from homogenized scraped nasal tissue. Subsequently, the cells were incubated on OXA-medium overnight at 35°C. Suspensions of fresh colonies of *S. aureus* 99308 containing \(3 \times 10^7–10^8\) cfu (in saline-150 mM NaCl) served as inocula for the intranasal infection in the mouse model.

**Determination of the MIC of mersacidin**

From mersacidin stock solutions in pure methanol (10 mg/mL), double serial dilutions in the range \(640–1.2\) mg/L in acetic acid (0.01% final concentration) supplemented with BSA (0.2% final concentration) were made.

The susceptibility of bacterial strains to mersacidin was evaluated\(^{22,23}\) on sterile U-shaped 96-well polystyrene microtitre plates (Costar Corp., Cambridge, MA, USA). Bacteria were grown overnight aerobically in Mueller–Hinton broth (MHB, Oxoid) at 35°C, subcultured to \(OD_{620}=0.2\), and diluted to obtain \(10^3–10^5\) cfu/mL in a final volume 0.1 mL. Serial dilutions of mersacidin were inoculated with bacterial suspensions in triplicate and incubated overnight. The MIC\(_{100}\) was defined as the lowest concentration of mersacidin inhibiting bacterial growth after 20 h of incubation at 35°C (S. aureus ATCC 25923, Escherichia coli ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains.

**Determination of the MBC of mersacidin**

The bactericidal effect of mersacidin was tested with *S. aureus* 99308. Bacterial cells in the logarithmic growth phase were exposed to mersacidin concentrations equivalent to \(1 \times, 5 \times\) and \(10 \times MIC\) in MHB. Bacteria were cultured for 20 h. Viable cfu (cfu/mL) were determined after 0, 2, 4, 6, 8, 10 h of incubation.

**Viable bacteria assay**

This test was also used to determine the presence of viable bacterial cells in blood and tissue samples of infected animals. Nasal scrapings, liver, lungs and kidney samples were pre-washed in fresh sterile 0.07 M phosphate buffered saline pH 7.2 (PBS), then placed in 1 mL of PBS and homogenized. A series of 10-fold dilutions were performed, and 10 µL of each dilution was plated in triplicate on Iso-Sensitest agar (Oxoid) plates and on OXA-medium.\(^{24}\) The colonies were counted after overnight incubation at 35°C and expressed as \(\log_{10}\). The limit of detection was \(<10^3\) cfu/mL.

**Animals**

Female BALB/cA mice (\(n=152\), 2 months old and weighing 25–27 g were used. Animals were fed a standard rodent chow diet in a temperature-controlled room (23°C) on a 12 h light/dark cycle. Forty-six mice were used for validation of the infection model, four mice for nasal bacterial inocula preparation, 48 mice (24 in model A and 24 in model B) for *S. aureus* 99308 intranasal colonization and 54 mice were assessed in nine different control groups (Table 1). The Lund University Ethical Review Committee for Animal Experiments approved the study.

**Development of the animal model**

Attempts to establish an exclusive rhinitis/carryer model for *S. aureus* 99308 in healthy mice with natural immune status were unsuccessful.\(^{26}\) However, when the natural immunity of the animals had been impaired by administration of a corticosteroid, it was possible to standardize a dose-dependent infectious rhinitis/carryer *S. aureus* mouse model. The time schedules for the development of the infection model are shown in Table 1.

The immune response of the mice was suppressed by scubcutaneous injections of hydrocortisone (Hydrocortisone hemisuccinum 100, Polfa, PL, 100 mg/kg/day) at day 0 and day 4 (Table 1). In the short term experiment (A), *S. aureus* 99308 was applied intranasally on days 5, 7 and 9. Mersacidin intranasal treatment was performed twice a day for 3 days on days 10, 11 and 12. In the long-term model (B), *S. aureus* 99308 intranasal applications were carried out on days 5, 7, 9, 30, 32 and 34, and the mice were cured with mersacidin on days 35, 36 and 37. A single dose of mersacidin set at 47 mg/kg was applied in a volume of 2 × 10 µL to the respective nares.\(^{21}\)
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Table 1. Time schedule describing the mouse model for nasal carriage of S. aureus 99308 and the treatment with mersacidin

<table>
<thead>
<tr>
<th>Days and treatments</th>
<th>A</th>
<th>B</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
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<td>11</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>A</td>
</tr>
</tbody>
</table>

A, short-term experiment; B, long-term experiment.

Twenty-four hours after the last mersacidin administration, on days 13 and 38, respectively, the animals were sacrificed, and tissue samples taken. Blood and internal organs e.g. lungs, kidney, liver and spleen samples were tested for the presence of S. aureus; nasal epithelium was tested for carriage of S. aureus in control and experimental animals as well as in model validation experiments.

Model validation

Infectious dose selection. Validation of the model and selection of the infectious dose were performed according to the scheme presented in Table 2. Two different doses of MRSA were tested intranasally, intraperitoneally and intranasally after treatment with corticosteroid. The low dose, i.e. 10^6 cfu/mL containing 3 × 10^2–10^4 cfu of S. aureus 99308, was used in further experiments for intranasal infections. The high dose was 3 × 10^6–10^8 cfu.

Potential toxic effects of mersacidin were also tested in vivo. Six mice were administered 10 mg of mersacidin intranasally according to the scheme presented (Table 1) and six animals received only saline. Following sacrifice, livers were sampled and fixed in Bouin’s solution, then routinely dehydrated and embedded in paraffin. Sections were then cut (5 μm) and mounted on Polysine glass (Histolab, Goteborg, Sweden). For morphological examination haematoxylin and eosin staining was used.

Determination of serum bactericidal titre of the mice

Blood samples were collected without any anticoagulant 2 h following intranasal MRSA administration, or following application of mersacidin, hydrocortisone and saline or from non-infected animals. The serum samples were immediately exposed to the same volume of MHB (Oxoid) containing S. aureus 99308 at a concentration of 1.53 × 10^8 cfu/mL. After 20 h of aerobic incubation at 35°C, the number of cfu of each group was determined. Bacterial counts were also determined after incubation without any mouse serum supplementation (bacterial growth control).

Cytokine and nuclear factor κB (NF-κB) estimations

Cytokine interleukin (IL)-1β and free forms of tumour necrosis factor-α (TNF-α) were measured in mice blood by sandwich enzyme immunoassay (CytElisa Mouse IL-1β, CytElisa Mouse TNFα, Nordic Biosite AB, Täby, Sweden). NF-κB transcription was analysed in nuclear extracts of scraped epithelial tissues, by electrophoretic mobility shift assay. NF-κB consensus oligonuclear probe 5'-GGGACTTTCC-3' (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was labelled with [32P]ATP (Amersham Pharmacia Biotech, Uppsala, Sweden) by incubation with T4 polynucleotide kinase (Roche Diagnostics Scandinavia AB, Bromma, Sweden). Nuclear extracts after incubation with a probe were separated on 5% polyacrylamide gels in Tris-borate-EDTA buffer. Gels were subsequently dried and analysed by autoradiography (Hyperfilm MP, Amersham Pharmacia Biotech).

Statistics

The results from the different groups of mice were compared statistically using a Student’s t-test or two-way analysis of variance (ANOVA) and Tukey’s post hoc test (SigmaStat for Windows v2.0, SPSS Science, Chicago, IL, USA).

Results

Antibacterial activity of mersacidin: MIC determination and mersacidin bactericidal effects in vitro

The MRSA strain S. aureus 99308 was selected for use in the in vivo model because the inflammatory immune response that is stimulated by this strain has been well characterized. The MIC of mersacidin against this strain was 1 mg/L. In order to estimate the bactericidal effect of mersacidin, a suspension of 10^6 cfu/mL of S. aureus 99308 was exposed to mersacidin at concentrations of 1, 5 and 10 mg/L (equivalent to 1 ×, 5 × and 10 × MIC), respectively. The bactericidal effect of the peptide was rapid and dose-dependent. Depending on the mersacidin concentration (10, 5, and 1 mg/L), viable bacteria were not detected after 2, 6 and 20 h of incubation. The time–kill curves are shown in Figure 2.

Validation of the animal model

Human S. aureus 99308 maintained its dose- and site-dependent virulence potential after adaptation to the mouse host (Table 2).

Table 2. Ratio between numbers of mice tested to mice successfully colonized or infected with S. aureus 99308

<table>
<thead>
<tr>
<th>S. aureus 99308</th>
<th>Intranasal infection</th>
<th>Intraperitoneal infection</th>
<th>Hydrocortisone + intranasal infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 × 10^2–10^4 cfu</td>
<td>6/0</td>
<td>6/0</td>
<td>6/6</td>
</tr>
<tr>
<td>3 × 10^4–10^6 cfu</td>
<td>6/0</td>
<td>6/6/6</td>
<td>6/6</td>
</tr>
</tbody>
</table>

*Lethal MRSA infections in three mice.*
No local or systemic effects were detected when a low dose of bacteria \((3 \times 10^{2}–10^{4} \text{ cfu})\) was applied intranasally or intraperitoneally in the mice (Table 2). The higher bacterial dose \((3 \times 10^{6}–10^{8} \text{ cfu})\) caused lethal infections after intraperitoneal application, whereas no colonization or general infection was noticed after intranasal inoculation. Subcutaneous treatment of the mice with the immunosuppressive agent hydrocortisone prior to intranasal inoculation of the MRSA at low and high doses resulted in successful infections of differing degrees. A low MRSA dose (inoculum \(3 \times 10^{2}–10^{4} \text{ cfu}\)) caused only colonization of nasal epithelial cells, whereas a high MRSA dose \((3 \times 10^{6}–10^{8} \text{ cfu})\) resulted in general infection. MRSA were isolated not only from nasal scrapings, but also from lung, spleen, kidney and liver samples. Thus, a low inoculum \((3 \times 10^{2}–10^{4} \text{ cfu})\) of \(S.\ aureus\) 99308 was set up as a standard to develop a rhinitis infection in mice pre-treated with hydrocortisone.

Mersacidin curative effects

Mersacidin administered intranasally twice daily \((1.66 \text{ mg/kg per treatment})\) over 3 days eradicated MRSA from the nasal mucosa of colonized mice (Table 3). The effectiveness of mersacidin treatment was independent of colonization time and the number of inoculations. Rhinitis caused by MRSA established after three inoculations over 5 days, or after six inoculations over 30 days (Table 1) did not alter the effectiveness of mersacidin (data not shown). The host immunity was impaired by hydrocortisone, as indicated by the decreased antibacterial activity of the sera of hydrocortisone-treated mice (see below) (Figure 3, bar 5). Before mersacidin administration, \(7.5 \times 10^{2}\) to \(1.5 \times 10^{3} \text{ cfu}\) of \(S.\ aureus\) 99308 were identified in nasal scrapings of 12 mice tested. In samples from mersacidin-treated animals, no \(S.\ aureus\) were recovered. In further tests, no infections were induced by tissue samples of mersacidin convalescent mice, homogenized and injected intraperitoneally into healthy animals (data not shown). No bacteraemia was observed in animals infected (day 6 and/or 31) and then treated with mersacidin (day 9 and/or 34).

Cytotoxicity tests showed that intranasal application of mersacidin in the same concentration as that used for treatment caused neither mucosal lesions nor morphological changes in the liver (data not shown).

\(S.\ aureus\) 99308 induced production of the pro-inflammatory cytokines IL-1\(\beta\) and TNF-\(\alpha\) (Table 3). The concentrations of the cytokines in blood after the bacterial infection amounted to \(60 \pm 0.1\) and \(280 \pm 1.8 \text{ pg/mL}\). In samples derived from animals cured with mersacidin, these cytokines were not detected \((0.0 \pm 0.6; 0.0 \pm 0.9 \text{ pg/mL},\) respectively). The data are mean ± S.D. of triplicate samples.

### Table 3. Influence of intranasal application of mersacidin on carriage of \(S.\ aureus\) 99308

<table>
<thead>
<tr>
<th></th>
<th>Before mersacidin treatment ((n = 12))</th>
<th>After mersacidin treatment ((n = 12))</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRSA in nasal cavity</td>
<td>(7.5 \times 10^{4}) to (1.5 \times 10^{5})</td>
<td>NF</td>
</tr>
<tr>
<td>Bacteraemia</td>
<td>NF</td>
<td>NF</td>
</tr>
<tr>
<td>Bacteria in internal organs</td>
<td>NF</td>
<td>NF</td>
</tr>
<tr>
<td>Serum IL-1(\beta)</td>
<td>(60 \text{ pg/mL} \pm 0.1) S.D.</td>
<td>(0.0 \text{ pg/mL} \pm 0.6) S.D.</td>
</tr>
<tr>
<td>Serum TNF-(\alpha)</td>
<td>(280 \text{ pg/mL} \pm 1.8) S.D.</td>
<td>(0.0 \text{ pg/mL} \pm 0.9) S.D.</td>
</tr>
<tr>
<td>NF-(\alpha)B — nasal epithelium</td>
<td>NF</td>
<td>NF</td>
</tr>
</tbody>
</table>

NF, not found.

\(\text{aBelow detection limit.}\)

### Influence of mice sera on growth of \(S.\ aureus\) 99308

The inhibitory potency of serum samples from control and infected and/or mersacidin-treated mice on the growth of \(S.\ aureus\) 99308 is presented in Figure 3, bars 1–10. MRSA growth in the incubation medium (Figure 3, bars 3 and 4) was significantly inhibited \((P < 0.05)\) by serum from control, non-infected mice. Serum samples derived from non-infected animals that had been treated intranasally with mersacidin revealed the greatest inhibitory effect on MRSA growth (Figure 3, bar 10). The number of bacterial cells dropped four \(\log_{10}\) below \((P < 0.001)\) the count of the control culture (Figure 3, bar 2).

Sera collected from mice infected for 30 days with MRSA restricted bacterial growth by three \(\log_{10}\) \((P < 0.001)\) compared with the control (bar 6). This antibacterial effect was identical.
to that exhibited by sera of control mice treated with hydrocortisone (Figure 3, bar 5). Serum samples from mice infected for 5 days (Figure 3 bar 7) demonstrated an additional significant decrease of bacterial cells (P<0.001) compared with the group infected with Staphylococcus aureus 99308 for 30 days (Figure 3, bar 6). This value is comparable to the inhibitory effect of sera from mice infected with the MRSA for 30 days and treated with mersacidin (Figure 3, bar 8). Sera from mice infected for a short time and treated with mersacidin (Figure 3, bar 9) generated the greatest inhibition of growth of Staphylococcus aureus 99308, comparable to the inhibition caused by sera from healthy mice treated with mersacidin.

Discussion

Treatment of Staphylococcus aureus infections may be complicated by multiple antibiotic resistances and specific virulence factors, causing temporary or long-lasting carriage. The nasal carriage of MRSA is a main risk factor for community-acquired infections and in hospital settings (nosocomial sepsis). Epidemiological data indicate that there are connections between MRSA nasal carriage and the development of staphylococcal infections. The infection rate in carriers of Staphylococcus aureus is higher than in non-carriers, and it has been well documented that humans are usually infected with their own nasal isolate. MRSA nasal carriage is usually treated with multiple applications of mupirocin nose salve; however, an increasing resistance against mupirocin, which is in part associated with prolonged use, emphasizes an urgent need for alternatives, particularly for hospital applications.

Antimicrobial peptides not only of host origin (innate defence effectors) but also compounds produced by the microflora (bacteriocin-like substances) could be an alternative for medical applications, especially considering that the mode of action of this group of peptides differs from classical antibiotics. In the early 1990s, a bacteriocin with good antimicrobial activity was identified (M 87–1551) from Bacillus sp. HIL Y-85, 54728 (FH 1658). This novel antibiotic peptide was active against different clinical staphylococcal strains including those isolated from blood and was called mersacidin due to its bactericidal activity against MRSA strains.

The eradication of MRSA from the nasal epithelium with mersacidin was the main objective of this study. To this end, a mouse model with corticosteroid (hydrocortisone) pre-treatment was successfully developed (Table 1). Double pre-treatment of the mice with hydrocortisone ensured a lower immunological status of the mice and allowed stable colonization of the MRSA in the nasal cavity (Table 2) after inoculation of a low dose of a bacteria in suspension. The infection model proposed in this study was suitable for in vivo evaluation of mersacidin action on MRSA carriage. Both the course of the MRSA infection and the curative effects of mersacidin were reproducible. Staphylococcus aureus 99308 has the ability to up-regulate its adhesion molecules and induces expression of E-selectin and ICAM-1 in human endothelial cells. However, the adhesive ability of this strain on non-human epithelium has not been examined (in particular some clinical isolates of MRSA carrying a type I SCC mec cassette are defective in cell attachment). Therefore, we tried to enhance the production of the bacterial polysaccharide capsule, which can contribute to cell–cell interaction in a specific manner. It is generally assumed that the capsule-producing cells adhere to certain matrices more easily. In order to ensure the induction of the bacterial infection in mice, Staphylococcus aureus 99308 was passed through the mouse host.

In the human Caucasian histiocytic lymphoma cell line U 937, Staphylococcus aureus 99308 can activate NF-κB and trigger interleukin synthesis (Å. Ljungh, unpublished results). In the present study, the Staphylococcus aureus 99308 strain did not activate NF-κB in vivo (Table 3), i.e. in nasal epithelium of MRSA-infected mice and from infected animals subsequently treated with mersacidin. In blood samples, it was possible to detect IL-1β only in infected mice that had not been treated with mersacidin. The missing reaction of the immune system is a reliable sign of the curative effect of mersacidin. In contrast, another lantibiotic, nisin, has not been successful so far in a similar rodent model, whereas the glycyglycyl endopeptidase lysostaphin seems to be useful in this approach.

The curative effect of mersacidin on rhinitis and the type of administration in this study highlight interesting objectives that should be further explored: (i) epithelial absorption and elimination curves, (ii) potential binding ability to serum proteins and (iii) finally the question of the site of mersacidin action—blood versus epithelium. Our studies indicate that mersacidin is absorbed into the bloodstream. Technically, our mouse infection model did not allow long-term monitoring of drug delivery from nasal epithelium to body fluids in a single animal and the possibility that mersacidin applied epithelially is active in the serum needs to be further investigated.

In summary, mersacidin was an effective drug with which to eradicate a nasal human MRSA strain adapted to mice. The mouse model developed in these experiments was successful for establishing an intranasal infection in mice with MRSA. The model proposed in this study is novel and suitable for in vivo evaluation of mersacidin action on MRSA carriage.

Acknowledgements

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References

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