Squalamine ointment for *Staphylococcus aureus* skin decolonization in a mouse model

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Received 22 November 2010; returned 5 January 2011; revised 11 February 2011; accepted 24 February 2011

**Objectives:** *Staphylococcus aureus* colonization of the skin and the nostrils remains a major cause of surgical-site infections despite preoperative and preventive procedures. To date, many compounds have been used for *S. aureus* decolonization, including mupirocin ointments and antiseptics, with variable results. The emergence of mupirocin-resistant *S. aureus* strains has led to the search for new antimicrobial agents specifically for *S. aureus* decolonization. In this work we evaluated squalamine and related parent-derived ointments (1%) as potential new compounds for *S. aureus* decolonization in a new mouse model.

**Methods:** We report the development and application of squalamine and related parent-derived ointments in a new mouse skin model. After skin shaving, mice were colonized with an *S. aureus* suspension that was calibrated to 10^4 – 10^6 cfu/mL. The remaining bacterial load was monitored for 2 days after a single application of squalamine by spreading.

**Results:** We found that *S. aureus* colonization of the skin was stable for at least 2 days before it was naturally eliminated. Using this model we found that squalamine ointment (1%) could reduce *S. aureus* viable cells by up to 4 log with a single, 1 h application of ointment, whereas mupirocin application reduced viable cell numbers by only 1.3 log during that same time (*P* < 0.05).

**Conclusions:** Our results suggest that such compounds may be useful for *S. aureus* nasal and skin decolonization and may constitute a potent alternative for skin and nasal antisepsis before surgery.

**Keywords:** aminosterol derivatives, *S. aureus*, mouse skin model

**Introduction**

*Staphylococcus aureus* is a human pathogen that colonizes multiple body sites such as the skin and nasal mucous membranes.1 *S. aureus* colonization constitutes a risk factor for patients undergoing surgery.2 Indeed, vascular and osteoarticular post-operative infections are often transmitted by asymptomatic chronic carriers with mucosal reservoirs, especially in the nasal mucosa.3 Thus, preoperative skin and nasal antiseptic strategies remain the most common and imperative medical preventive procedures to reduce post-surgical infections.9 In past decades, antibiotics such as rifampicin, mupirocin and fusidic acid have been widely used to eradicate *S. aureus* colonization of the nostrils and the skin.5–7 Nevertheless, antibiotic resistance, especially mupirocin resistance, which was first reported in 1987, has now been reported in many cases.5,10,11 An alternative to mupirocin is lysostaphin cream; lysostaphin is an antibiotic that cleaves the cross-linking pentaglycine bridges in *Staphylococcus* cell walls. Lysostaphin cream has been shown to be very efficient compared with mupirocin in a mouse model of *S. aureus* nasal decolonization.12 Other compounds, such as antiseptics, have also been used, including chlorhexidine alcohol and povidone-iodine administered with mupirocin, with rates of up to 75% methicillin-resistant *S. aureus* (MRSA) decolonization in human patients.13,14 The major advantage for the use of antiseptics is their ability to possess broad-spectrum activity13 and reduce the risk of surgical infection in hospitals, as well as acting on multiple cellular targets. Their major disadvantage is they are flammable alcohol-based products. Because of the emergence and spread of resistance to these compounds, there is a need to develop new alternative compounds for preoperative preventive measures for *S. aureus* decolonization.

In this context, squalamine (Figure 1), a water-soluble natural polyaminosterol isolated from the tissues of the dogfish shark (*Squalus acanthias*) has shown a wide spectrum of antimicrobial activity, especially against *S. aureus*.15 The synthesis of squalamine is complicated and yields insufficient output.16 We were able to demonstrate that numerous synthetic aminosterol...
derivatives, such as aminosterol derivative 2 (ASD-2), possess similar, or better, antistaphylococcal activity against a large panel of S. aureus clinical isolates from the sputa of cystic fibrosis patients. Moreover, the dramatic physical effect of squalamine against S. aureus, as recently described, may represent an advantage for the development of such compounds for topical use. The aim of our study was to evaluate and compare the well-known antibiotic squalamine and an aminosterol derivative, which were both added individually to ointments, as potential new drugs for rapid S. aureus decolonization in a new mouse skin model.

Materials and methods

Compounds

Squalamine was a gift from Professor M. Zasloff (Georgetown University, Washington, DC). ASD-2 was synthesized according to the previously reported procedure (Figure 1). Stock solutions (2 g/L) of squalamine and ASD-2 were prepared in water and methanol, respectively. Stock solutions were subsequently diluted in water to 250 mg/L working solutions. The antibiotic controls used in this study were vancomycin (Merck Généraux, Lyon, France), mupirocin ointment (Bactroban 2%; GlaxoSmithKline Laboratory, France) and fusidic acid ointment (Fucidine 2%; Leo Laboratory, France).

Bacterial strains

Reference bacterial strains used were methicillin-susceptible S. aureus (ATCC 25923) and MRSA CF Marseille (CSUR P102). Susceptibility testing MICs were determined in duplicate by the broth microdilution method, conducted according to BSAC guidelines, providing a limit of detection of 60 cfu/mL. Bacteria were cultivated on Trypticase soy agar (TSA) plates for 24 h at 37°C.

In vitro selection of aminosterol-resistant S. aureus

Squalamine and ASD-2 were successively diluted in 5 mL of Mueller–Hinton medium to different concentrations ranging from 0.5–4 times the MIC. Suspending MICs were determined in duplicate by the broth microdilution method, conducted according to BSAC guidelines, providing a limit of detection of 60 cfu/mL. Bacteria were cultivated on Trypticase soy agar (TSA) plates for 24 h at 37°C. Absence of bacterial contamination in the different ointments was verified by suspending them in sterile distilled water, depositing 100 μL of each suspension on a TSA plate and incubating the plate at 37°C for 24 h. Absence of any bacterial growth after 48 h of culture incubation was considered a marker of ointment sterility.

Preparation of aminosterol and vancomycin ointments

The aminosterol and vancomycin ointments (1%) were prepared by mixing 9.9 g of petrolatum-based cream (Cooper, Cooperation Pharmaceutique Francé, France) and 100 mg of the desired active substance. Briefly, 100 mg of squalamine or the aminosterol derivative were weighed and crushed in a mortar. Petrolatum-based cream was added gradually and the contents were mixed for 10 min until a homogeneous cream was obtained. Finally, the ointment was stored in a sterile tube at 4°C. Absence of bacterial contamination in the different ointments was verified by suspending them in sterile distilled water, depositing 100 μL of each suspension on a TSA plate and incubating the plate at 37°C for 24 h. Absence of any bacterial growth after 48 h of culture incubation was considered a marker of ointment sterility.

Mouse model of S. aureus skin colonization

Cutaneous colonization was studied exclusively in 4–8-week-old female BALB/c mice; inbred strain (Charles River Laboratory, France). Each mouse received with a tested compound was carried out with five mice in two independent experiments. Mice were anaesthetized with ketamine (25 mg/kg) and xylazine (3 mg/kg) (Bayer and Panpharma Laboratory, respectively), and a 9 cm² area on the back dorsal cervical surface was shaved with an electric razor. The shaved area was divided into two equal zones.

In the first set of experiments, 10 μL of a bacterial suspension of S. aureus calibrated to 10⁶ cfu/mL was applied to the skin of the shaved mice on the two different zones. After 1 h, 20 mg of pure petrolatum-based cream (control) or the aminosterol-derivative ointment was spread in single application on the left and right zones, respectively. For some experiments, antibiotic ointments were used in place of the aminosterol-derivative ointments. Colonization was monitored by swabbing the skin of the mice once daily using a dry swab (sterile swab, wooden applicator and cotton tip; Capan Italia S.p.A., Brescia, Italy) after 0, 24, 48 and 72 h. The swabs were inoculated in 1 mL of physiological serum, 10-fold serial dilutions were performed and 100 μL of each dilution was plated on Chapman agar plates. These plates were incubated at 37°C for 24 h before bacterial enumeration (cfu/mL).

In a second set of experiments, 10 μL of a S. aureus bacterial suspension calibrated to 10⁶ cfu/mL was applied to the skin of shaved mice, as described above. After 1 h, 20 mg of pure petrolatum-based cream (control) or cream containing an antibiotic (vancomycin, fusidic acid or mupirocin) or the aminosterol derivative ASD-2 was spread in a single application on the left and right zones, respectively. For some experiments, antibiotic ointments were used in place of the aminosterol-derivative ointments. Colonization was monitored by swabbing the skin of the mice once daily with a dry swab (sterile swab, wooden applicator and cotton tip; Capan Italia S.p.A., Brescia, Italy) after 0, 24, 48 and 72 h. The swabs were inoculated in 1 mL of physiological serum, 10-fold serial dilutions were performed and 100 μL of each dilution was plated on Chapman agar plates. These plates were incubated at 37°C for 24 h before bacterial enumeration (cfu/mL).

Selection of mupirocin-resistant S. aureus

Selection of a mupirocin-resistant strain of S. aureus was performed using the CF Marseille S. aureus strain (CF Marseille CSUR P102), which was available in our laboratory. Susceptibility to mupirocin was estimated by culturing the bacteria in the presence of mupirocin discs (5 μg); the diameters of the zones of growth inhibition were measured after incubation at 37°C for 24 h.

After 24 h, colonies that had grown around the disc were resuspended in 3 mL of Luria–Bertani broth and incubated for 3 h at 37°C. The suspension was calibrated to 10⁶ cfu/mL and spread on TSA plates, and a mupirocin disc was deposited in the centre of the plate. This procedure was repeated until complete resistance to mupirocin developed, i.e. observation of growth in contact with the mupirocin discs.

Figure 1. Structures of squalamine and ASD-2.
Table 1. MIC values of squalamine, ASD-2 and classical antibiotics for S. aureus strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>Squalamine MIC (mg/L)</th>
<th>ASD-2 MIC (mg/L)</th>
<th>Mupirocin MIC (mg/L)</th>
<th>Vancomycin MIC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. aureus ATCC 25923</td>
<td>2</td>
<td>2</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>S. aureus CF Marseille CSUR P102</td>
<td>2</td>
<td>2</td>
<td>0.5</td>
<td>1</td>
</tr>
<tr>
<td>S. aureus CF Marseille CSUR P102, mupirocin-resistant</td>
<td>2</td>
<td>2</td>
<td>100</td>
<td>0.5</td>
</tr>
</tbody>
</table>

application, as previously described. Colonization was monitored after 0, 30 and 60 min, and bacteria were counted as described above.

This study (registry number: 2-28012011) was approved by our local Ethics Committee (Faculty of Medicine, Université de la Méditerranée, Marseille, France).

Results

As shown in Table 1, squalamine and ASD-2 possess similar activity against susceptible and methicillin-resistant CF Marseille S. aureus strains, with 2 mg/L MIC values in all cases. We were not able to select for an in vitro aminosterol-resistant S. aureus strain even after having repeated the experiment more than 20 times. Conversely, a mupirocin-resistant S. aureus isolate was obtained by in vitro selection on plates with a mupirocin disc after 33 subcultures. The initial mupirocin MIC, as determined using the microdilution method, was 0.5 mg/L, whereas the fully resistant isolate had an MIC value of 100 mg/L. Interestingly, the MIC values of squalamine and ASD-2 were the same for this mupirocin-resistant strain. Squalamine, ASD-2 and vancomycin ointments (1%) were prepared extemporaneously for each experiment, and the sterility of each cream was verified by microbiological analyses that demonstrated no bacterial growth.

For the mouse skin model, brief anaesthesia was necessary to shave the mice properly and to apply the bacterial suspension. In the first set of experiments we applied 10 μL of a bacterial suspension (10⁶ cfu/mL) to the skin to monitor the natural elimination of bacteria over 3 days. Bacterial enumerations during this challenge showed that the bacterial load remained stable for at least 2 days (10⁷ cfu/mL) and then naturally decreased to 10⁷ cfu/mL by day 3 (Figure 2a). Thus we studied the efficacy of squalamine and aminosterol ASD-2 ointment on S. aureus colonization over the course of 3 days. A single application of squalamine and ASD-2 ointment was able to reduce S. aureus viable cells by up to 4 log compared with the control (P<0.05) 2 days after application. In the second set of experiments we studied and compared the rapidity of action of squalamine and ASD-2 with various antibiotics for skin decolonization after 30 and 60 min (Figure 2b). Interestingly, a single squalamine ointment application was able to reduce the bacterial load of S. aureus viable cells by 4 log after only 30 min. Fusidic acid, mupirocin and ASD-2 reduced the bacterial population by 1.7, 1.3 and 1 log after 1 h, respectively, under the same experimental conditions (Figure 2b). Using a vancomycin ointment (1%), no reduction in bacterial growth was observed after 1 h. This result was similar to that obtained with the control. Finally, no significant differences were noticed in terms of the appearance of treated or untreated skin patches, and no lesions or inflammation were encountered after the application of aminosterol-derivative ointments.

Discussion

Testing antistaphylococcal therapeutics for skin and/or nasal decolonization generally requires a suitable animal model. Several animal models for nasal colonization have been previously reported, but such models are usually time consuming and labour intensive. Thus these models are not suitable for the rapid screening of new compounds. Moreover, it has been demonstrated that mouse nasal colonization models are not easy to implement, and high levels of colonization could not be achieved using such models. These models have also been shown to be inconsistent and not reproducible. The cotton rat model has been used as an alternative model for nasal colonization and has proven to be reproducible with higher levels of persistent S. aureus nasal colonization, i.e. bacterial loads ranging from 10⁵–10⁶ cfu/nose that may persist up to 6 weeks. However, this model is very fastidious, since it requires intranasal S. aureus instillation, animal treatment, nostril bisection with scissors and an S. aureus culture that is not suitable for rapid screening. This is the reason why we investigated the
development of an easier and faster mouse skin model to screen our compounds. We found that our model was easy to implement by just shaving mice and applying bacterial suspensions and ointments directly to the skin. This model is efficient, as it yields a reproducible level of Staphylococcus aureus colonization, ranging from $10^4$–$10^6$ cfu/mL over the course of 2 days, comparable to levels in the cotton rat model. Natural elimination of bacteria occurred after 3 days in our model. It is well known that staphylococcal skin infections often clear spontaneously, with a peak in S. aureus charge at 2–4 days and a rapid decline thereafter. For this reason, our mouse skin model cannot be used as a chronic S. aureus colonization model, but rather as a rapid test to screen potential active compounds.

Squalamine has reached Phase III trials for the treatment of age-related macular degeneration and prostate cancer disease without any major side effects, as it appears to be well tolerated even at doses 250 mg/day in adults, suggesting that an ointment for local use could be totally safe. Comparisons between the petrolatum-based cream (control), squalamine and ASD-2 ointment revealed a high degree of bacterial load reduction (up to 4 log, bactericidal effect) of viable S. aureus cells during a 2 day experiment. These results suggest the possible use of these ointments for S. aureus decolonization. Moreover, we found that squalamine rapidly killed bacteria, with a single application of ointment reducing bacterial load by up to 4 log after only 30 min. Fusidic acid is known to have little or no bactericidal effect on S. aureus in vitro, whereas a 2 log reduction for mupirocin and a 3 log reduction for vancomycin have been reported after 24 h in a time-kill study assay. In the study by LaPlante, bacterial load reductions at 4 h for mupirocin and fusidic acid were less than 1 log. The rapid bactericidal effect of squalamine could be attributable to the dramatic depolarizing effect observed on S. aureus, which resulted in rapid cell death. Although ASD-2 possesses the same in vitro activity against S. aureus as squalamine, ASD-2 does not decolonize S. aureus-colonized skin as quickly as squalamine. In terms of time efficiency, this difference may be due to the structure of the aminosterol derivative or, more probably, its diffusion ability (ASD-2

Conclusions

In summary, our mouse skin colonization model may be a valuable tool for screening compounds that may have potent activity for S. aureus skin decolonization. With this model we have demonstrated that squalamine in a cream base may be an alternative to mupirocin ointment for the eradication of S. aureus skin colonization. Finally, our results support the development of such creams for the rapid eradication of S. aureus skin and nasal colonization for the prevention of nosocomial S. aureus infections. Nevertheless, even if these data are promising, they still remain preliminary in nature.

Acknowledgements

We acknowledge Mr Claude Napppez and Ms Linda Hadjadj for their experimental assistance.

Funding

This work was supported by the Centre National de la Recherche Scientifique (France).

Transparency declarations

None to declare.

References


