Polyaminosteroid Analogues as Potent Antibacterial Agents Against Mupirocin- Resistant *Staphylococcus aureus* Strains

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Title: Polyaminosteroid derivatives as potent antibacterial agents against mupirocin-resistant *Staphylococcus aureus* strains

Running title: Squalamine analogue efficacy in resistant *Staphylococcus aureus* strains

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29 **Summary**

30 Background and Objective:

31 Nasal carriage of *Staphylococcus aureus* (*S. aureus*) constitutes an important risk factor for
32 subsequent infections in some types of patient populations. Decolonization of carriers using
33 intranasal mupirocin is widely used as a preventive measure. Resistance to this agent has
34 been rising and causing failure in the decolonization and thus in the prevention of infections.

35 Methods and Results:

36 In this study, we demonstrate that squalamine and one of its analogues, named BSQ-1, are
37 active against mupirocin-susceptible and -resistant clinical isolates belonging to *S. aureus*
38 species. Additionally, long-term and repeated exposure of a susceptible *S. aureus* strain to
39 squalamine and BSQ-1 do not lead to the emergence of resistant bacteria, contrarily to
40 mupirocin, suggesting the potent interest of their use as decolonizing agents.

Introduction

Nasal carriage of *Staphylococcus aureus* (*S. aureus*), including methicillin resistant *S. aureus* (MRSA) is considered as a risk factor for subsequent staphylococcal infections [1–3]. This was mainly demonstrated in patients undergoing orthopaedic or cardiac surgeries [2,3], dialysis [4], and patients in intensive care units [5]. Among a series of medical preventive measures, decolonization of *S. aureus* using intranasal mupirocin was shown to be effective [6–8]. However, resistance to mupirocin has been rising and causing treatment failures [9–11]. Thus, there is a need to develop new and highly effective drugs with low propensity for resistance induction, to ultimately replace mupirocin in this indication. In this context, squalamine (Figure 1), a natural aminosterol extracted from the tissues of the dogfish shark, has demonstrated potent antibacterial, antifungal, antiviral and antiangiogenic activities [12–15]. However, the synthesis of squalamine is complex with limited yields and leads to the design of squalamine analogues that are easily synthesized [16,17]. This class of molecules acts in a unique mechanism of action against both Gram-positive and Gram-negative bacteria, without emergence of resistance [16][18]. The objective of our study was to evaluate the antibacterial activity of squalamine and one of its analogues (BSQ-1) (Figure 1) against *S. aureus* clinical strains with different levels of mupirocin-resistance.

Materials and methods

Bacterial strains

Sixteen *S. aureus* strains were randomly selected in a collection of *mupA*-positive and -negative clinical isolates that were previously collected from nasal carriers hospitalized in Croix Rousse Hospital (Hospices Civils de Lyon, Lyon, France) [19].

Presence of *mupA*, *mupB* and *mecA* genes had been screened among this collection using multiplex *mupA/mecA*/16S and *mupB*/16S PCR as described elsewhere [20]. The *mupB*-positive isolate was kindly provided by Roberto Melano (University of Toronto, Canada). *S. aureus* strain ATCC 25923 (methicillin-susceptible *S. aureus*, MSSA) [21], *S. aureus* strain CF Marseille CSUR P102 (methicillin-resistant *S. aureus*, MRSA) [22], and the mupirocin-resistant/MRSA strain CSUR P1946 were used as reference.

Antibacterial agents

Squalamine was synthesized according to a previously published procedure [23–25]. BSQ-1 was provided by Biosqual SAS according to a procedure previously reported in our laboratory [16]. Mupirocin powder was purchased from Sigma Aldrich (Saint Louis, Missouri, USA).

Five milligrams of squalamine or BSQ-1 powders were weighed and dissolved in water or in DMSO for mupirocin, to have stock solutions at a 5 mg/mL concentration. Further dilutions were done in water to obtain a final solution at 444 µg/mL used in our experiments.

Broth microdilution

Minimum inhibitory concentrations (MICs) of tested molecules were determined by the broth microdilution method according to the CLSI guidelines [26]. In 96-well plates, 90 µL of Mueller Hinton II broth (cation-adjusted) (MHB) (Becton Dickinson, Le Pont de Claix, France) were put in all the wells and 90 µL of the antibacterial solution at 444 µg/mL was added to the first well. Further two-fold dilutions were done by transferring 90 µL of the mixed solution in the first well to the subsequent wells containing MHB. Overnight bacteria were used to prepare a microbial suspension adjusted to 0.5 McFarland that was further

diluted 1:20. Ten microliters of this suspension were added to the two-fold dilutions of antibacterial agents to obtain a final volume of 100 μ L containing 5×10^5 cfu/mL in each well. Plates were incubated at 37°C for 24 h and read to determine the MICs. The MIC was defined as the lowest concentration of antimicrobial agent that inhibits visible growth of the organism in the wells. Experiments were done in triplicate.

Sequencing of mupA gene

Sequencing of the *mupA* gene was done only when the gene was found by PCR and the strain was susceptible to mupirocin in the broth microdilution method. PCR amplification products that were positive for the *mupA* gene were sequenced using BigDye terminator chemistry on an automated ABI 3130 sequencer (PE Applied Biosystems, Foster City, CA). Sequenced genes were compared to other genes using the BlastN and BlastP tools from the National Centre for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov). Then the genes were compared to the reference *mupA* gene of strain *EU658880.1 Staphylococcus epidermidis* using the nps alignment software (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html).

Resistance induction

The potential of squalamine and BSQ-1 to induce resistant strains was evaluated on the ATCC 25923 strain. Bacteria were cultured in MHB with constant shaking at 160 rpm at 37°C. The next day, 1 mL of the inoculum was adjusted to 10^5 cfu/mL and 1 mL of antibacterial agents were added at a final concentration of half the MIC. Tubes were incubated again at 37°C for 24h. Subculture was then done in drug-free MHB and incubated in the same conditions. Again, a suspension calibrated at 10^5 cfu/mL was inoculated into

broth containing half the MIC. This was repeated daily for 10 series of exposition-subculture. After these expositions, the MIC was re-evaluated by the broth microdilution method as described above [27]. If the value of the MIC remained unchanged, we considered that the bacteria did not develop resistance. If the value of the MIC had risen, we considered that the bacteria developed resistance to the corresponding agent.

Results

We tested a total of 16 *S. aureus* strains among which nine harboured the *mupA* gene and one the *mupB* gene. For strains exhibiting mupirocin resistance genes, the MIC of this molecule was $> 200 \mu\text{g/mL}$, except for the ST 20141927 that was susceptible to mupirocin (MIC = $0.195 \mu\text{g/mL}$) despite the detection of the *mupA* gene using PCR. Sequencing of this latter revealed that a nucleotide deletion (G228del) leading to a frameshift mutation, resulting in the formation of a stop codon TAG in the *mupA* gene. This will lead to the formation of a truncated polypeptide made of 79 amino acid residues.

Squalamine and BSQ-1 were both effective against all bacterial strains regardless of the presence of resistance genes for mupirocin, with MICs values of $3.125 \mu\text{g/mL}$ (Table 1).

Attempts to induce resistance were performed by exposing 10 times the ATCC 25923 strain to subinhibitory concentrations of squalamine, BSQ-1 and mupirocin independently. It was observed that the MICs of squalamine and BSQ-1 remained unchanged after these 10 expositions whereas the MIC increased from $0.39 \mu\text{g/mL}$ to $12.5 \mu\text{g/mL}$ after 7 expositions to mupirocin, highlighting the emergence of resistance in the tested *S. aureus* strain (Table 2).

Discussion

Until now, mupirocin is considered as the gold standard for nasal decolonization of *S. aureus* [28]. This antibiotic inhibits the bacterial isoleucyl-tRNA synthetase (IRS) and subsequently protein synthesis, leading to bacterial death [29]. The efficacy of intranasal mupirocin to prevent subsequent staphylococcal infections has been mainly demonstrated in surgical settings [6][29]. In 2016, the World Health Organization published recommendations on preoperative measures for the prevention of surgical site infections [31]. Thus, preoperative decolonization of *S. aureus* nasal carriers undergoing cardiothoracic and orthopaedic procedures with intranasal mupirocin, with or without chlorhexidine body wash was strongly recommended [31].

In this context, it is noteworthy that resistance to mupirocin has been first reported in 1987 [32] and is still rising worldwide [10]. For instance, a recent German study demonstrated that the prevalence of *mupA* gene encoding high-level mupirocin resistance in a tertiary care centre in Saxony reached nearly 20% in 2016-2017 after being below 1% from 2000-2015 [33].

Two levels of mupirocin resistance have been described : a low-level mupirocin resistance (LLMR) defined by a MIC $\geq$ 8–256 μ g/mL, which is due to point mutations in the native IRS gene (*ileS*) and a high-level mupirocin resistance (HLMR) defined by a MIC $\geq$ 512 μ g/mL, which is due to the acquisition of a plasmid-mediated gene, *mupA*, (*ileS*₂), encoding a modified IRS with less affinity for mupirocin [34]. In addition to the *mupA* gene, a novel locus, named *mupB*, has been also recently reported as a mechanism for acquisition of HLMR [35] (even if a single isolate has been reported until now).

Presence of HLMR or combined LLMR and chlorhexidine resistance have been associated with decolonization failure and increase in the risk of persistent *S. aureus* carriage [9][11][35] suggesting that alternatives to these prophylactic drugs are needed.

In the present study, we show that squalamine and BSQ-1 are active against clinical *S. aureus* strains involved in nasal carriage even if resistant to mupirocin. In staphylococcal strains that harboured the *mupA* or *mupB* gene, mupirocin presented a MIC of > 200 µg/mL. Of note, the strain ST 20141927 despite carrying the *mupA* gene presented a MIC towards mupirocin of 0.195 µg/mL due to genetic deletion. Such a phenomenon dealing with the presence of the *mupA* gene and mupirocin susceptibility was described in previous studies as well [37][38].

In a second time, we investigated the potential of the different molecules to induce resistance in the ATCC 25923 *S. aureus* strain. After exposing 10 times this strain to subinhibitory concentrations of squalamine and BSQ-1, the MIC of these molecules remained unchanged (3.125 µg/mL) indicating that no resistance emerged. Put together with recently published data concerning the peculiar mechanism of action of polyaminosterol such as squalamine, that acts in a detergent-like manner [39], this strongly pinpoints that BSQ-1 would be unable to lead for selecting resistant strains [18]. Conversely, the same *S. aureus* strain exposed to mupirocin using the same protocol exhibited a 32-times increase in mupirocin MIC after only 7 runs of exposition, highlighting the development of resistance. In agreement with our results, Lee *et al.* showed that *in-vitro* incubation with subinhibitory concentrations of susceptible MRSA strains to mupirocin for 7 to 14 days, is sufficient to induce mutations in *ileS* gene leading to low-level mupirocin resistance [27].

Conclusion

In conclusion, our findings indicate that squalamine and its analogue BSQ-1 are active against all tested mupirocin-susceptible and resistant clinical *S. aureus* strains involved in nasal carriage. In the tested experimental conditions, we demonstrated that these new agents did not lead to the emergence of resistant strains, unlike mupirocin. While the resistance to mupirocin is still rising and being a source of treatment failures, squalamine and BSQ-1

appear as promising new alternatives for nasal decolonization to prevent endogenous infection, limit transmission and fight resistant pathogens. Further studies are now under investigation to evaluate their activity and tolerance in animal models before clinical trials.

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Conflict of interest/Transparency declaration

Squalamine analogues are under development by the start-up Biosqual.

Jean-Marc Rolain and Olivier Blin are shareholders at Biosqual and Adèle Sakr is an employee at the start-up.

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341 Figures

344 **Figure 1.** Structure of squalamine and BSQ-1

345

Tables

Table 1. MICs ($\mu\text{g/mL}$) of squalamine, BSQ-1 and mupirocin against clinical strains of *S. aureus*.

Strain	Gene	BSQ-1	Squalamine	Mupirocin
20151051	<i>mupA</i>	3.125	3.125	>200
20150729	<i>mupA</i>	3.125	3.125	>200
20150549	<i>mupA</i>	3.125	3.125	>200
20141927	<i>mupA</i>	3.125	3.125	0.195
20141143	-	3.125	3.125	0.39
20140465	<i>mupA</i>	3.125	3.125	>200
20120179	<i>mupB</i>	3.125	3.125	0.195
20140890	-	3.125	3.125	0.195
20141488	-	3.125	3.125	0.39
20150635	<i>mupA</i>	3.125	3.125	>200
20150099	-	3.125	3.125	0.39
20141075	<i>mupA</i>	3.125	3.125	>200
20150771	-	3.125	3.125	0.39
20141759	<i>mupA</i>	3.125	3.125	>200
20142157	<i>mupA</i>	3.125	3.125	>200
20141351	-	3.125	6.25	0.39
<i>S. aureus</i> ATCC 25923		3.125	3.125	0.39
<i>S. aureus</i> CSUR P102		3.125	3.125	0.39
<i>S. aureus</i> CSUR P1946	<i>mupA</i>	3.125	3.125	>200

353 Table 2. MIC ($\mu\text{g/mL}$) values showing the variation of susceptibility of *S. aureus* strain
 354 ATCC 25923 after 7 expositions to the molecules BSQ-1, squalamine or mupirocin.

		BSQ-1	Squalamine	Mupirocin
ATCC	25923	3.125	3.125	0.39
original strain				
ATCC	25923	3.125	3.125	12.5
exposed	to			
mupirocin				
ATCC	25923	3.125	3.125	0.39
exposed	to			
squalamine				
ATCC	25923	3.125	3.125	0.39
exposed to BSQ-1				

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357 **Tables and figures**

358 **Tables: 2**

359 **Table 1.** MICs ($\mu\text{g/mL}$) of squalamine, BSQ-1 and mupirocin against clinical strains of *S.*
360 *aureus*

361 **Table 2.** MIC ($\mu\text{g/mL}$) values showing the variation of susceptibility of *S. aureus* strain
362 ATCC 25923 after 7 expositions to the molecules BSQ-1, squalamine or mupirocin:

363

364 **Figures: 1**

365 **Figure 1.** Structure of squalamine and BSQ-1

366