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

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1 **Title:** Polyaminosteroid derivatives as potent antibacterial agents against mupirocin-resistant  
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3 **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.

## 41 **Introduction**

42 Nasal carriage of *Staphylococcus aureus* (*S. aureus*), including methicillin resistant *S. aureus*  
43 (MRSA) is considered as a risk factor for subsequent staphylococcal infections [1–3]. This  
44 was mainly demonstrated in patients undergoing orthopaedic or cardiac surgeries [2,3],  
45 dialysis [4], and patients in intensive care units [5]. Among a series of medical preventive  
46 measures, decolonization of *S. aureus* using intranasal mupirocin was shown to be effective  
47 [6–8]. However, resistance to mupirocin has been rising and causing treatment failures [9–  
48 11]. Thus, there is a need to develop new and highly effective drugs with low propensity for  
49 resistance induction, to ultimately replace mupirocin in this indication. In this context,  
50 squalamine (Figure 1), a natural aminosterol extracted from the tissues of the dogfish shark,  
51 has demonstrated potent antibacterial, antifungal, antiviral and antiangiogenic activities [12–  
52 15]. However, the synthesis of squalamine is complex with limited yields and leads to the  
53 design of squalamine analogues that are easily synthesized [16,17]. This class of molecules  
54 acts in a unique mechanism of action against both Gram-positive and Gram-negative bacteria,  
55 without emergence of resistance [16][18]. The objective of our study was to evaluate the  
56 antibacterial activity of squalamine and one of its analogues (BSQ-1) (Figure 1) against *S.*  
57 *aureus* clinical strains with different levels of mupirocin-resistance.

58

## 59 **Materials and methods**

### 60 *Bacterial strains*

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

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

70

#### 71 *Antibacterial agents*

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

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

79

#### 80 *Broth microdilution*

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

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

93

#### 94 *Sequencing of mupA gene*

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

104

#### 105 *Resistance induction*

106 The potential of squalamine and BSQ-1 to induce resistant strains was evaluated on the  
107 ATCC 25923 strain. Bacteria were cultured in MHB with constant shaking at 160 rpm at  
108 37°C. The next day, 1 mL of the inoculum was adjusted to  $10^5$  cfu/mL and 1 mL of  
109 antibacterial agents were added at a final concentration of half the MIC. Tubes were  
110 incubated again at 37°C for 24h. Subculture was then done in drug-free MHB and incubated  
111 in the same conditions. Again, a suspension calibrated at  $10^5$  cfu/mL was inoculated into

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

117

## 118 **Results**

119 We tested a total of 16 *S. aureus* strains among which nine harboured the *mupA* gene and one  
120 the *mupB* gene. For strains exhibiting mupirocin resistance genes, the MIC of this molecule  
121 was > 200 µg/mL, except for the ST 20141927 that was susceptible to mupirocin (MIC =  
122 0.195 µg/mL) despite the detection of the *mupA* gene using PCR. Sequencing of this latter  
123 revealed that a nucleotide deletion (G228del) leading to a frameshift mutation, resulting in  
124 the formation of a stop codon TAG in the *mupA* gene. This will lead to the formation of a  
125 truncated polypeptide made of 79 amino acid residues.

126 Squalamine and BSQ-1 were both effective against all bacterial strains regardless of the  
127 presence of resistance genes for mupirocin, with MICs values of 3.125 µg/mL (Table 1).

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

133

## 134 **Discussion**

135 Until now, mupirocin is considered as the gold standard for nasal decolonization of *S. aureus*  
136 [28]. This antibiotic inhibits the bacterial isoleucyl-tRNA synthetase (IRS) and subsequently  
137 protein synthesis, leading to bacterial death [29]. The efficacy of intranasal mupirocin to  
138 prevent subsequent staphylococcal infections has been mainly demonstrated in surgical  
139 settings [6][29]. In 2016, the World Health Organization published recommendations on  
140 preoperative measures for the prevention of surgical site infections [31]. Thus, preoperative  
141 decolonization of *S. aureus* nasal carriers undergoing cardiothoracic and orthopaedic  
142 procedures with intranasal mupirocin, with or without chlorhexidine body wash was strongly  
143 recommended [31].

144 In this context, it is noteworthy that resistance to mupirocin has been first reported in 1987  
145 [32] and is still rising worldwide [10]. For instance, a recent German study demonstrated that  
146 the prevalence of *mupA* gene encoding high-level mupirocin resistance in a tertiary care  
147 centre in Saxony reached nearly 20% in 2016-2017 after being below 1% from 2000-2015  
148 [33].

149 Two levels of mupirocin resistance have been described : a low-level mupirocin resistance  
150 (LLMR) defined by a MIC  $\geq$  8–256  $\mu$ g/mL, which is due to point mutations in the native IRS  
151 gene (*ileS*) and a high-level mupirocin resistance (HLMR) defined by a MIC  $\geq$  512  $\mu$ g/mL,  
152 which is due to the acquisition of a plasmid-mediated gene, *mupA*, (*ileS2*), encoding a  
153 modified IRS with less affinity for mupirocin [34]. In addition to the *mupA* gene, a novel  
154 locus, named *mupB*, has been also recently reported as a mechanism for acquisition of  
155 HLMR [35] (even if a single isolate has been reported until now).

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



159 In the present study, we show that squalamine and BSQ-1 are active against clinical *S. aureus*  
160 strains involved in nasal carriage even if resistant to mupirocin. In staphylococcal strains that  
161 harboured the *mupA* or *mupB* gene, mupirocin presented a MIC of > 200 µg/mL. Of note, the  
162 strain ST 20141927 despite carrying the *mupA* gene presented a MIC towards mupirocin of  
163 0.195 µg/mL due to genetic deletion. Such a phenomenon dealing with the presence of the  
164 *mupA* gene and mupirocin susceptibility was described in previous studies as well [37][38].  
165 In a second time, we investigated the potential of the different molecules to induce resistance  
166 in the ATCC 25923 *S. aureus* strain. After exposing 10 times this strain to subinhibitory  
167 concentrations of squalamine and BSQ-1, the MIC of these molecules remained unchanged  
168 (3.125 µg/mL) indicating that no resistance emerged. Put together with recently published  
169 data concerning the peculiar mechanism of action of polyaminosterol such as squalamine,  
170 that acts in a detergent-like manner [39], this strongly pinpoints that BSQ-1 would be unable  
171 to lead for selecting resistant strains [18]. Conversely, the same *S. aureus* strain exposed to  
172 mupirocin using the same protocol exhibited a 32-times increase in mupirocin MIC after only  
173 7 runs of exposition, highlighting the development of resistance. In agreement with our  
174 results, Lee *et al.* showed that *in-vitro* incubation with subinhibitory concentrations of  
175 susceptible MRSA strains to mupirocin for 7 to 14 days, is sufficient to induce mutations in  
176 *ileS* gene leading to low-level mupirocin resistance [27].

177

## 178 **Conclusion**

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

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

187

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194

### 195 **Conflict of interest/Transparency declaration**

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

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

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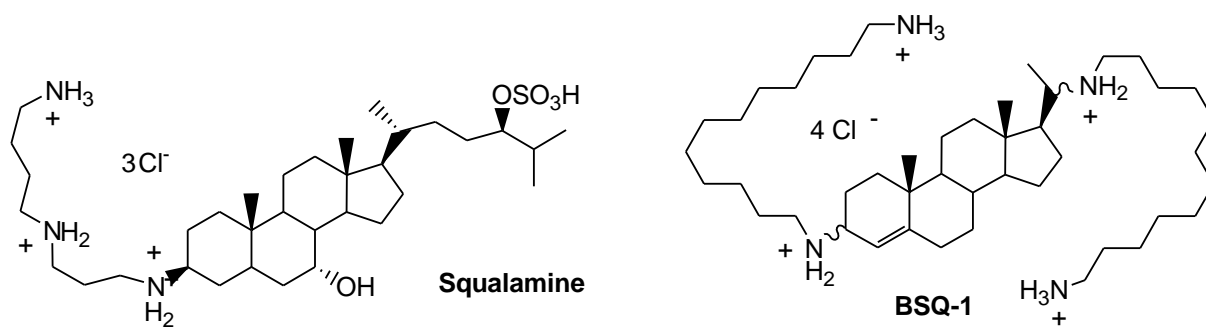
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341 Figures

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

345

346 Tables

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

348 *aureus*.

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

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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.

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

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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:

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364 **Figures: 1**

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

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