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1 **Anti-persister activity of squalamine against *Acinetobacter baumannii***

2

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22 **Keywords:** *A. baumannii*, squalamine, persister, VBNC, anti-persister.

23

24 **ABSTRACT**

25 Squalamine is a natural polycationic aminosterol extracted from *Squalus acanthias*. It
26 displays a remarkable efficacy against Gram- and Gram+ bacteria which are resistant to
27 antimicrobials. Its membranolytic activity and its low cytotoxicity make squalamine one of
28 the most promising agents to fight against nosocomial pathogens such as *Acinetobacter*
29 *baumannii*. In the context of chronic diseases and therapeutic failures associated to this
30 pathogen, the presence of dormant cells, *i.e.* persisters and viable but not culturable cells
31 (VBNCs), highly tolerant to antimicrobial compounds is problematic. The aim of this study
32 was to investigate the antibacterial activity of squalamine against this bacterial population of
33 *A. baumannii*. Bacterial dormancy was induced by cold shocks and nutriment starvation in the
34 presence of high doses of either colistin, ciprofloxacin, or squalamine. Persisters and VBNCs
35 induced by these treatments were then challenged by 100 mg/L of squalamine. The efficacy of
36 each treatment was determined by evaluating the culturability on agar medium, the membrane
37 integrity (Live/Dead™ *BacLight*™ staining) and the respiratory activity (CTC *BacLight*™
38 RedoSensor™ staining) of bacteria. We showed that *A. baumannii* ATCC 17978 generated
39 persisters as well as VBNCs in the presence of high doses of ciprofloxacin whereas colistin
40 and squalamine did not. We demonstrated that squalamine at 100 mg/L (below its hemolytic
41 concentration) was able to kill dormant cells. Squalamine did not induce persisters nor
42 VBNCs formation in *A. baumannii* ATCC 17978. Interestingly, it was significantly active
43 against this type of dormant population generated by ciprofloxacin, making it a very
44 promising anti-persister agent.

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46

47 **INTRODUCTION**

48 *Acinetobacter baumannii* is a nosocomial pathogen responsible for many infections usually
49 localized on skin and in urinary or respiratory tracts. The emergence of multi-drug resistance
50 in this bacterium obviously complicates the treatment efficiency¹. However, the resistant
51 character of *A. baumannii* is not the sole cause of therapeutic failure. Indeed, dormant cells,
52 *i.e.* persisters and Viable But Non Culturable cells (VBNCs), are known to survive to high
53 amounts of antimicrobials, and play an important role in the infection relapse and chronic
54 diseases².

55 Unlike resistance, dormancy is a reversible physiological state initiated by multiples
56 environmental stresses. It is often associated with a slow or arrested growth and a reduced
57 metabolic activity allowing the development of an antibiotic resistant phenotype while
58 maintaining a genome similar to that of a susceptible strain². Among the different pathways
59 contributing to the formation of this dormant population, the activation of toxin-antitoxin
60 systems mediated by (p)ppGpp second messenger (alarmones guanosine pentaphosphate), is
61 the most widely described^{2,3}. Persisters and VBNCs have been proposed to be associated
62 within a continuum of dormancy, and would be mainly differentiated by the culturability of
63 persisters when stressful conditions are removed, whereas VBNCs required revivification
64 step³.

65 Today, few options to eradicate these dormant cells are available since most treatments have
66 been developed to eradicate active populations. Among the main strategies to kill persister
67 cells remain²: i) the use of molecules, like antimicrobial peptides or small molecule which
68 target cell membrane, that do not require a metabolically active target, and ii) the metabolic
69 reactivation of dormant cells to restore susceptibility to classical treatments, using, for
70 example, saccharides that were showed to enhance the proton motive force of the bacterial
71 membrane. Unfortunately, any of these studies were tested for their anti-VBNCs activity.

72 In *A. baumannii*, the phenomenon of dormancy has not been extensively studied. Actually,
73 persister cells of *A. baumannii* were induced by high doses of antibiotics belonging to β -
74 lactamines^{4,5}, polymyxins⁶ and also aminosides^{5,7} families. The size of persister population
75 appeared also variable depending of the strain and the antibiotic used^{4,7}. Unlike other bacteria
76 from the ESKAPE group, *A. baumannii* persisters did not have a cross-tolerance: they were
77 exclusively persistent for the compound inducing the dormancy⁸. Moreover, if the ability to
78 enter in VBNC state was established in *A. baumannii*⁹, it was not examined concomitantly
79 with persistence. So far, any anti-persister molecules have been proposed to eradicate this
80 population in *A. baumannii*.

81 Isolated from the shark *Squalus acanthias*¹⁰, squalamine is a polycationic aminosterol which
82 possesses antiangiogenesis, antiobesity, antiviral, antifungal as well as antibacterial
83 properties^{11,12}. In this latter case, squalamine has been demonstrated to act as a membranolytic
84 molecule against a wide panel of both susceptible and resistant Gram+ or Gram- bacteria.
85 Furthermore, even though its bactericidal effect has been demonstrated on numerous
86 pathogens, squalamine has still not been tested on persisters and VNBCs populations.

87 In the present study, we have generated dormant populations of *A. baumannii*, using cold
88 shocks, nutriment deprivation in the presence of high amounts of ciprofloxacin and
89 demonstrated that squalamine displayed an efficient activity against these recalcitrant cells.

90 **MATERIALS AND METHODS**

91 *Isolation of dormant cells*

92 Dormant cells (persisters and VBNCs) were generated and isolated as described by Marques
93 *et al.*¹³. Briefly, an *A. baumannii* ATCC 17978 culture was grown in Mueller Hinton Broth
94 (MH Broth, Difco) at 37°C overnight. It was then diluted at 1% in fresh medium and
95 incubated for additional 24 h. Bacteria were recovered by centrifugation at 5 000 × *g* for 5
96 min at 4°C and washed twice with cold 0.85% NaCl (saline solution, Sigma Aldrich, USA).
97 Resulting pellet was re-suspended in cold saline solution at an optical density of 0.8 at 600
98 nm, and incubated with increasing concentrations (0-500 mg/L) of colistin or ciprofloxacin
99 (Sigma Aldrich, USA), at 37°C for 48 h. After cells washing with 0.1 M Phosphate Buffered
100 Saline solution (pH 7.4, PBS, Fisher Sc., USA), plate numerations were performed in
101 triplicate on MH Agar and read at different incubation times from 0 to 48 h to determine cell
102 densities (CFU/mL). Determination of antimicrobial minimal inhibitory concentration (MIC)
103 was performed by microdilution method before and after persister isolation step. Each
104 experiment was performed in triplicate.

105

106 *Effect of squalamine on dormant populations*

107 The effect of squalamine was tested on dormant populations generated and isolated after a
108 treatment by ciprofloxacin or colistin at 500 mg/L, respectively named CIP500 and COL500,
109 as described in the previous section. After a 48 h of these first antibiotic treatments, cells were
110 harvested by centrifugation (5 000 × *g* for 5 min at 4°C). Pellets were suspended in a ½
111 volume of saline solution and cells were incubated at 37°C for additional 48 h with either 500
112 mg/L ciprofloxacin (CIP/CIP500) to confirm the dormancy, or 500 mg/L colistin
113 (CIP/COL500), or 100 mg/L squalamine¹⁴ (CIP/SQ100) to test their activity on dormant cells.
114 Similarly, cells isolated after a first COL500 treatment were incubated with either 500 mg/L

115 of colistin (COL/COL500) or 100 mg/L of squalamine (COL/SQ100). Finally, cells isolated
116 after a first 100 mg/L of squalamine (SQ100) were treated by additional 100 mg/L squalamine
117 treatment as control (SQ/SQ100). All cell density (CFU/mL) were enumerated as described
118 above. All experiments were performed in triplicate.

119

120 *Flow cytometry and staining methods*

121 One milliliter of each cell suspension (CIP500, COL500, SQ100, CIP/CIP500, CIP/COL500,
122 CIP/SQ100, COL/COL500, COL/SQ100, SQ/SQ100) were normalized at 10^6 CFU/mL and
123 stained in the dark by 3 μ L of Live/Dead *BacLight* kit (50/50 v/v, Live/DeadTM *BacLight*TM
124 Viability/cytotoxicity Kit, Molecular Probes-Invitrogen) for 30 min at room temperature or by
125 100 μ L of 5-cyano-2,3-ditolyltetrazolium chloride (CTC, *BacLight*TM RedoSensorTM CTC
126 Vitality Kit, Molecular Probes-Invitrogen) at 50 mM for 2 h at 37°C. Cells were then counted
127 by BD AccuriTM C6 flow cytometer (Becton Dickinson, CA, USA) with a low flow at 30
128 μ L/min. Statistical analysis of bacterial populations obtained before and after treatment was
129 performed using ANOVA one way test. All quantifications were performed in triplicate.

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134 **RESULTS AND DISCUSSION**

135 *Isolation of dormant cells*

136 It generally is assumed that within bacterial cultures, a stochastic population of dormant cells
137 including persisters is generated². In addition, dormant cells can also be induced by several
138 environmental stresses like starvation, oxidative stress, pH, carbon-source transitions,
139 antibiotics...³. The persister population in *A. baumannii* cultures represent usually a low
140 fraction of cells (< 1%) like in several bacterial species^{4,7}. Thus, in order to accurately
141 estimate the squalamine efficiency on *A. baumannii* dormant cells, we aimed to increase the
142 size of this subpopulation (persisters and VBNCs). For this purpose, we followed the protocol
143 described by Marques *et al.*¹³. We also tested three growth conditions: (i) the concentration of
144 antibiotics (colistin and ciprofloxacin at concentrations ranging from 0 to 500 mg/L), (ii) the
145 nature of the culture medium (MHB or saline solution) as well as (iii) the incubation time (24
146 h or 48 h).

147 Regarding colistin treatment, the analysis of the culturability (proving the existence of
148 persisters) showed that, under these stressful growth conditions, *A. baumannii* was highly
149 tolerant to colistin below 10 mg/L (corresponding to 20-fold of MIC = 0.5 mg/L) whatever
150 the culture medium used (Figure S1). This result was consistent with a recent study⁶ pointing
151 out the presence of a tolerant population in the presence of 10 mg/L colistin. However, this
152 subpopulation disappeared after a 24 h treatment with increasing colistin doses for 50 or 250
153 mg/L, in MHB and saline medium respectively. It should be noticed that the saline medium
154 promoted more *A. baumannii* culturability for high colistin doses than the rich medium tested.
155 Concerning ciprofloxacin, persisters were present at the highest concentration of 500 mg/L
156 (*i.e.*, 1000 x of MIC = 0.5 mg/L) (Figure S2). The percentage of culturable/persister cells was
157 again influenced by the nature of the culture medium with (0.54±0.41)% and

158 (0.00001±0.000015)% of the population after 48 h of incubation in saline medium and MHB,
159 respectively.

160 Taken together, these results suggest as already observed for *Pseudomonas aeruginosa*¹³, that
161 the persistent state in *A. baumannii* is promoted by stresses, *e.g.*, nutriment deprivation (saline
162 medium) and cold shock. Even though a colistin treatment is often associated to a tolerance
163 period in some ESKAPE bacteria¹⁵, our result indicate that it does not generate persister
164 population whereas this population was currently isolated with ciprofloxacin^{13,16}. The same
165 conclusions could be drawn for our results on *A. baumannii*.

166 Thus, we next generated persisters by a 500 mg/L ciprofloxacin treatment (CIP500) in saline
167 medium for 48 h to investigate ~~test~~ the anti-persisters activity of squalamine. This incubation
168 time was chosen since at this time the population is the most homogenous (Figure S2).
169 Ciprofloxacin MIC on the remaining bacterial population (0.5 mg/L) was similar to the initial
170 population MIC, thus demonstrating the presence of persisters but not resistant cells.
171 Treatment by colistin at 500 mg/L (COL500), in the same conditions of growth, was used as a
172 negative control (Figure S2). CIP500 and COL500 cultures were then analyzed by a
173 Live/Dead *BacLight* staining to evaluate the membrane integrity of the cell population and by
174 a CTC staining to explore the cell respiratory activity. Analyses of the COL500 culture, which
175 presented no culturable cells, showed that still (2.73±0.52)% of remaining cells retained a
176 respiratory activity and (0.70±1.15)% of them a membrane integrity. The corresponding cells
177 may be VBNCs remaining after colistin treatment (Figure 1B, Table 1). However, these small
178 values have to be compared to those obtained after an autoclaved culture and could also
179 reflect the residual fluorescence noise (Table 1).

180 Regarding CIP500 culture, while only (0.43±0.11)% of the initial population remained
181 culturable ((2.08±0.13).10⁵ CFU/mL, Figure 1A&B), a large subpopulation, *i.e.*,
182 (87.73±3.15)% conserved its membrane integrity and (45.83±5.98)% also exhibited a

183 respiratory activity (Figure 1B, Table 1). Thus it remained an important cell population still
184 respiring but non-culturable. This population may be VBNCs that have not been eradicated by
185 ciprofloxacin. As already described, high-dose of antibiotic treatment can indeed induce both
186 persister and VBNC populations, the latter being far more abundant up to 100-fold¹⁷.
187 We can notice a higher percentage of viable cells by Live/Dead staining compared to CTC.
188 Such an overestimation of living cells in combined SYTO9/PI staining was already reported
189 and may originate from PI background fluorescence as well as a higher affinity of SYTO9 for
190 dead cells than living ones¹⁸.
191 Finally, a culture grown in the same conditions treated with 100 mg/L squalamine (*i.e.*, 50 x
192 of MIC = 2 mg/L) was performed. Squalamine did not generate persisters (Figure 1A). No
193 cell exhibit a membrane integrity and only (2.73±0.12)% of them were still respiring. These
194 values below the ones observed for autoclaved cells (Table 1) are promising for testing the
195 efficiency of squalamine against *A. baumannii* dormant cells.

196

197 ***Effect of squalamine on dormant cells***

198 After their isolation by a 48 h-ciprofloxacin treatment (Figure 1), dormant cells were treated,
199 in the same growth conditions, for additional 48 h, either with ciprofloxacin at 500 mg/L (to
200 confirm its inefficiency), or with 500 mg/L of colistin or 100 mg/L of squalamine¹⁴ to
201 evaluate the effect of these antimicrobials on dormant population (persisters and VBNCs).
202 The second treatment by ciprofloxacin induced a slight decrease of the number of culturable
203 cells similarly to the reduction for control population (CIP/CIP500, Figure 2). Analyses of the
204 bacterial population by flow cytometry confirmed that viable cells remained numerous after
205 this second treatment (18.63±3.26 % of the initial population still respiring, Table 1). In
206 contrast, the dormant population was significantly affected by colistin (CIP/COL500) and
207 squalamine (CIP/SQ100). These two membranolytic compounds eradicated persisters in less
208 than 3 and 1 h, respectively (Figure 2). In accordance, the percentages of cells keeping their

209 membrane integrity, *i.e.*, (1.90±0.99)% and (0.07±0.03)% for colistin and squalamine,
210 respectively, or their respiratory activity ((2.27±1.07)% and (1.77±0.58)% respectively), were
211 very low (Table 1). These data demonstrate that squalamine, like colistin, obviously kill *A.*
212 *baumannii* dormant cells.

213 Finally, a second treatment of the COL500 culture either with 500 mg/L of colistin
214 (COL/COL500) or 100 mg/L squalamine (COL/SQ100) and a double treatment with
215 squalamine (SQ/SQ100) were performed as controls. As shown in Table 1, only remaining
216 respiratory activity exists in the three cultures but percentages of viable cells remained under
217 values of the negative autoclaved control.

218 We demonstrated in this study that squalamine is able to eradicate the persisters isolated after
219 ciprofloxacin treatment. Recently, several bactericidal compounds were shown to exhibit an
220 anti-persister activity². These molecules, such as SPI009¹⁹ and (RW)₄-NH₂ antimicrobial
221 peptide²⁰, present the common characteristic, like squalamine¹⁵, to induce damages to
222 membrane, a non-metabolically active target. This mode of action allows also squalamine to
223 be efficient on VBNCs as do colistin. The concomitant study of both anti-persisters and anti-
224 VBNCs properties of squalamine is particularly valuable in a context of chronic infections.

225

226 **CONCLUSION**

227 Squalamine displayed a great efficiency against *A. baumannii* dormant cells (*i. e.* persisters
228 and VBNCs). Unlike for colistin, doses used here are far below the minimal hemolytic
229 concentration (MHC > 200 mg/L)¹⁶ and makes this natural compound a very promising agent
230 to fight against an infection relapse related to *A. baumannii* dormant cells. Further
231 investigations are under current analyze to decipher the mechanism of action involved in the
232 anti-persister/anti-dormancy activity of squalamine.

233

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237

238 **DECLARATIONS**

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241 **Competing Interests:** None

242 **Ethical Approval:** Not required

243

244

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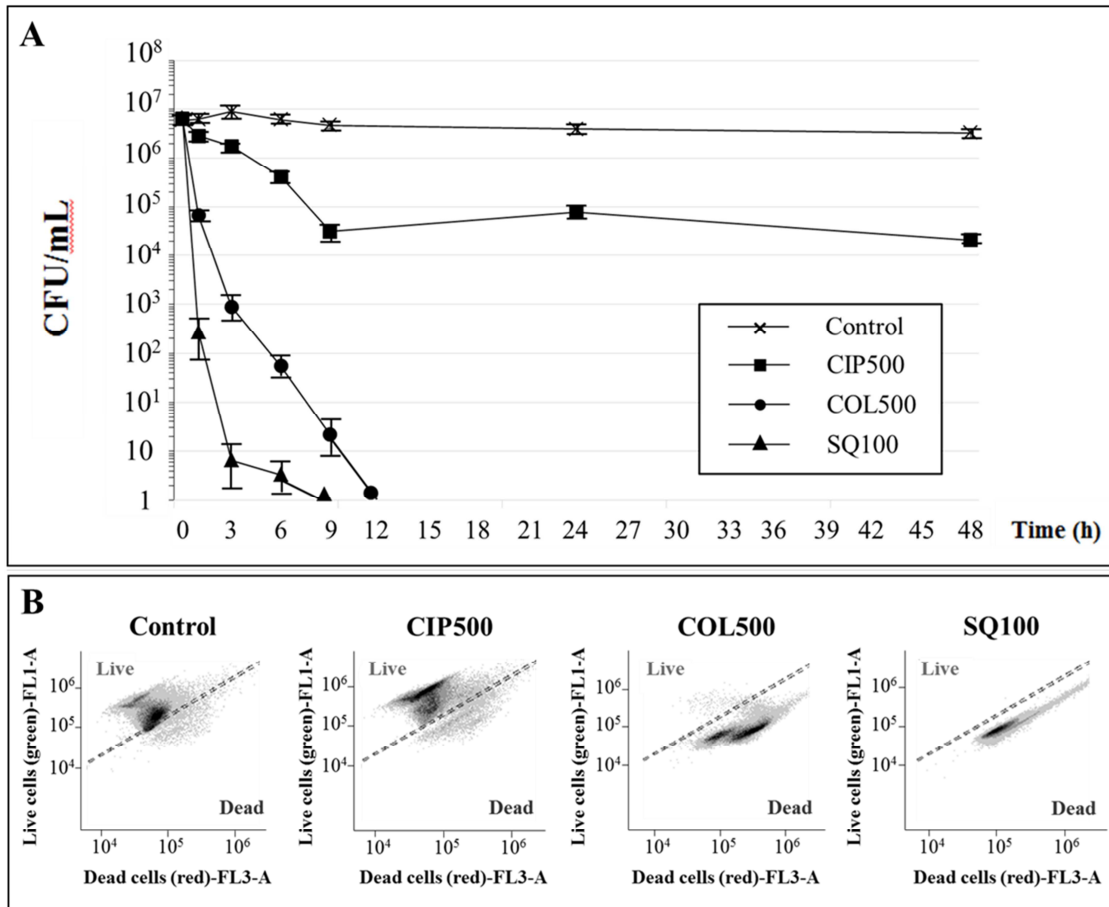


Figure 1 : Isolation of dormant populations in saline medium. (A) Culturability is given in CFU/mL depending on time (from 0 to 48 h) in presence of ciprofloxacin at 500 mg/L (filled squares, CIP500), colistin at 500 mg/L (filled circles, COL500) or squalamine at 100 mg/L (filled triangles, SQ100) or without any compound as control (cross, Control). **(B) Flow cytometry profiles after Live/Dead BacLight staining.** For each population (Control, CIP500, COL500 and SQ100), the dot blot represents the green (live) versus the red (dead) fluorescents corresponding to fractions of cells which have respectively retained or not their membrane integrity after treatment. Each population (CIP500, COL500 and SQ100) are significantly different ($p < 0.001$) compared to the initial population from 3h.

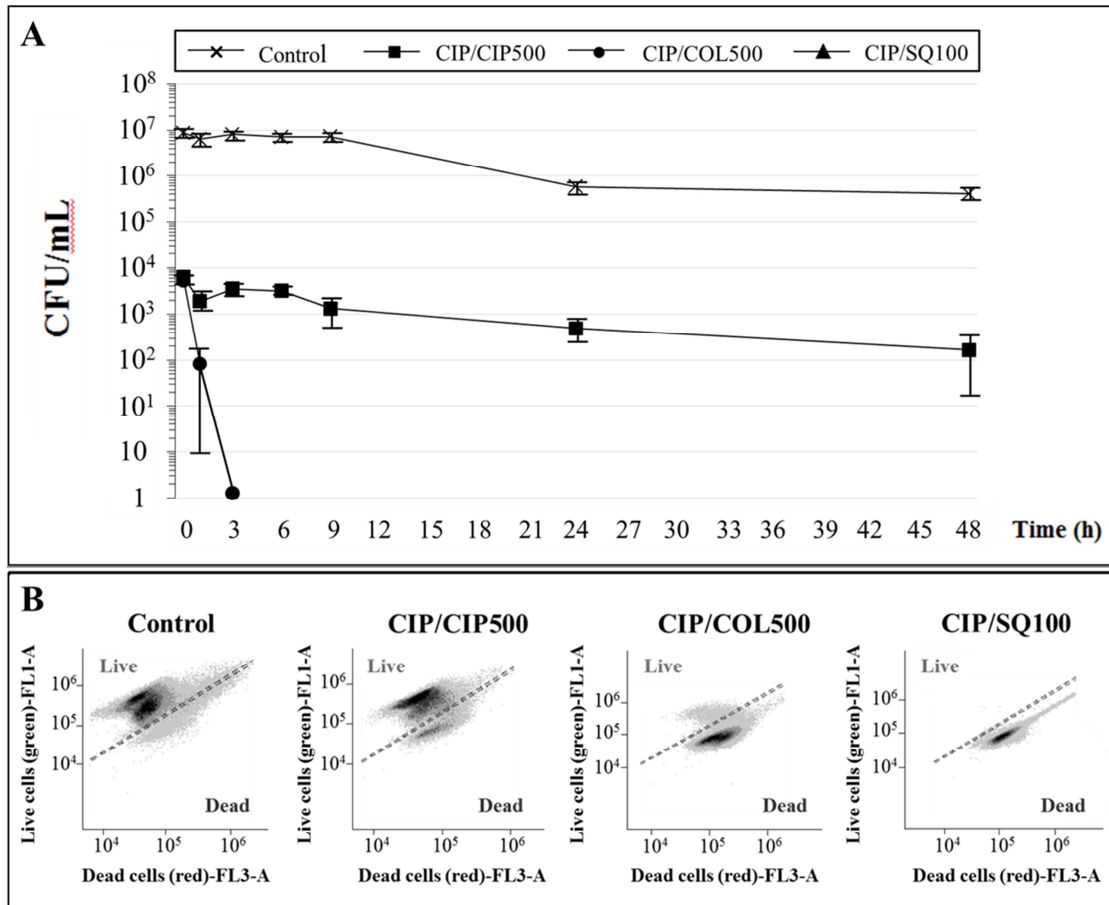


Figure 2: Effect of squalamine on dormant populations in saline medium. (A) Culturability is given in CFU/mL depending on time (from 0 to 48 h) after a first treatment with 500 mg/L ciprofloxacin. The resulted population was treated by 500 mg/L ciprofloxacin (filled squares, CIP/CIP500), 500 mg/L colistin (filled circles, CIP/COL500) or 100 mg/L squalamine (filled triangles, CIP/SQ100) or without any antibacterial compound as control (cross, Control). For CIP/SQ100 cultures, the culturability limit was reached before 1h of incubation. **(B) Flow cytometry profiles after Live/Dead BacLight staining.** For each population (Control, CIP/CIP500, CIP/COL500 and CIP/SQ100), the dot blot represents the green (live) versus the red (dead) fluorescents corresponding to fractions of cells which have respectively retained or not their membrane integrity after the second antibacterial treatment. The populations CIP/COL500 and CIP/SQ100 are significantly different ($p < 0.0001$) compared to the initial population from 1h.

Table 1: Analyses by flow cytometry of bacterial populations retaining membrane integrity (Live) or respiratory activity (Respiring) after antibacterial treatment.

		Live/Dead <i>BacLight</i>		CTC
		Live (%)	Dead (%)	Respiring (%)
Isolation	Autoclaved control	1.23 ± 0.43	91.87 ± 5.6	2.5 ± 0.53
	Control	86.27 ± 0.39	5.27 ± 1.02	66.13 ± 6.73
	COL500	0.70 ± 1.15	* ** * 98.40 ± 0.20	* ** * 2.73 ± 0.52
	CIP500	87.73 ± 3.15	10.10 ± 2.51	45.83 ± 5.98
	SQ100	0	99.17 ± 0.18	2.73 ± 0.12
Second treatment	Control	87.77 ± 4.78	9.90 ± 4.16	22.37 ± 4.03
	CIP/CIP500	76.53 ± 10.59	21.87 ± 10.27	18.63 ± 3.26
	CIP/COL500	1.90 ± 0.99	* ** * 96.93 ± 0.72	* ** * 2.27 ± 1.07
	CIP/SQ100	0.07 ± 0.03	99.53 ± 0.09	1.77 ± 0.58
	COL/COL500	4.10 ± 0.10	95.25 ± 0.05	1.65 ± 0.05
	COL/SQ100	0.10	99.3 ± 0.30	1.95 ± 0.05
	SQ/SQ100	0	99.85 ± 0.05	1.45 ± 0.05

*** for p value < 0.0001 ; ** for p value < 0.001

GRAPHICAL ABSTRACT

