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Short Communication

Anti-persister activity of squalamine against *Acinetobacter baumannii*

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ABSTRACT

Squalamine is a natural polycationic aminosterol extracted from the shark *Squalus acanthias*. Squalamine displays remarkable efficacy against antimicrobial-resistant Gram-negative and Gram-positive bacteria. Its membranolytic activity and low cytotoxicity make squalamine one of the most promising agents to fight nosocomial pathogens such as *Acinetobacter baumannii*. In the context of chronic diseases and therapeutic failures associated with this pathogen, the presence of dormant cells, *i.e.* persisters and viable but non-culturable cells (VBNCs), highly tolerant to antimicrobial compounds is problematic. The aim of this study was to investigate the antibacterial activity of squalamine against this bacterial population of *A. baumannii*. Bacterial dormancy was induced by cold shock and nutrient starvation in the presence of high doses of either colistin, ciprofloxacin or squalamine. Persisters and VBNCs induced by these treatments were then challenged with 100 mg/L squalamine. The efficacy of each treatment was determined by evaluating culturability on agar medium, membrane integrity (LIVE/DEAD® *Bac*Light™ staining) and respiratory activity (*Bac*Light™ RedoxSensor™ CTC staining) of bacteria. *A. baumannii* ATCC 17978 generated persisters as well as VBNCs in the presence of high doses of ciprofloxacin but not colistin or squalamine. Squalamine at 100 mg/L (below its haemolytic concentration) was able to kill dormant cells. Squalamine did not induce persister cell or VBNC formation in *A. baumannii* ATCC 17978. Interestingly, squalamine was significantly active against this type of dormant population generated by ciprofloxacin, making it a very promising anti-persister agent.

1. Introduction

Acinetobacter baumannii is a nosocomial pathogen responsible for many infections usually localised on the skin and in the urinary or respiratory tracts. The emergence of multidrug resistance in this bacterium complicates treatment options [1]. However, the resistant characteristic of *A. baumannii* is not the sole cause of therapeutic failure. Indeed, dormant cells, *i.e.* persisters and viable but non-culturable cells (VBNCs), are known to survive high concentrations of antimicrobials and to play an important role in infection relapse and chronic diseases [2].

Unlike resistance, dormancy is a reversible physiological state initiated by multiple environmental stresses. It is often associated with slow or arrested growth and reduced metabolic activity allowing the development of an antibiotic-resistant phenotype while maintaining a genome similar to that of a susceptible strain [2]. Among the different pathways contributing to the formation of this dormant population, the most widely described is activation of toxin–antitoxin systems mediated by (p)ppGpp second messenger (alarmone guanosine pentaphosphate) [2,3]. Persisters and VBNCs have been proposed to be associated within a continuum of dormancy and can be mainly differentiated by the culturability of persisters when stressful conditions are removed whereas VBNCs required a revivification step [3].

Today, few options are available to eradicate these dormant cells since most treatments have been developed to eradicate active populations. Among the main strategies to kill persister cells are [2] (i) the use of molecules such as antimicrobial peptides or small molecules targeting the cell membrane that do not require a metabolically active target and (ii) the metabolic reactivation of dormant cells to restore susceptibility to classical treatments, *e.g.* using saccharides that have been showed to enhance the protonmotive force of the bacterial membrane. Unfortunately, none of these strategies have been tested for their anti-VBNC activity.

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The phenomenon of dormancy has not been extensively studied in *A. baumannii*. Persister cells of *A. baumannii* are induced by high doses of antibiotics belonging to the β-lactam [4,5], polymyxin [6] and aminoglycoside [5,7] families. The size of the persister population appears to be variable depending on the strain and the antibiotic used [4,7]. Unlike other bacteria from the 'ESKAPE' group, *A. baumannii* persisters do not have cross-tolerance to other clinically important antibiotics but are exclusively persistent for the compound inducing the dormancy [8]. Moreover, although the ability to enter the VBNC state has been established in *A. baumannii* [9], it was not examined concomitantly with persistence. So far, any anti-persister molecules have been proposed to eradicate this population of *A. baumannii*.

Squalamine is a polycationic aminosterol isolated from the shark *Squalus acanthias* [10] that possesses anti-angiogenesis, anti-obesity, antiviral, antifungal and antibacterial properties [11,12]. In the latter case, squalamine has been demonstrated to act as a membranolytic molecule against a wide range both of susceptible and resistant Gram-positive and Gram-negative bacteria. Furthermore, although its bactericidal effect has been demonstrated on numerous pathogens, squalamine has not yet been tested on persister and VBNC populations.

In the present study, dormant populations of *A. baumannii* were generated using cold shock and nutrient deprivation in the presence of high concentrations of ciprofloxacin and it was demonstrated that squalamine displayed an efficient activity against these recalcitrant cells.

2. Materials and methods

2.1. Isolation of dormant cells

Dormant cells (persisters and VBNCs) were generated and isolated as described by Marques *et al.* [13]. Briefly, an *A. baumannii* ATCC 17978 culture was grown overnight in Mueller–Hinton broth (MHB) (Difco) at 37 °C. The culture was then diluted at 1% in fresh medium and was incubated for an additional 24 h. Bacteria were recovered by centrifugation at 5000 × *g* for 5 min at 4 °C and were washed twice with cold 0.85% saline solution (Sigma-Aldrich, USA). The resulting pellet was re-suspended in cold saline solution at an optical density at 600 nm of 0.8 and was incubated with increasing concentrations (0–500 mg/L) of colistin or ciprofloxacin (Sigma-Aldrich) at 37 °C for 48 h. After cell washing with 0.1 M phosphate-buffered saline solution (pH 7.4) (Thermo Fisher Scientific, USA), colonies on the plate were enumerated in triplicate on Mueller–Hinton agar read at different incubation times from 0–48 h to determine cell densities (CFU/mL). Determination of the antimicrobial minimal inhibitory concentration (MIC) was performed by the microdilution method before and after the persister isolation step. Each experiment was performed in triplicate.

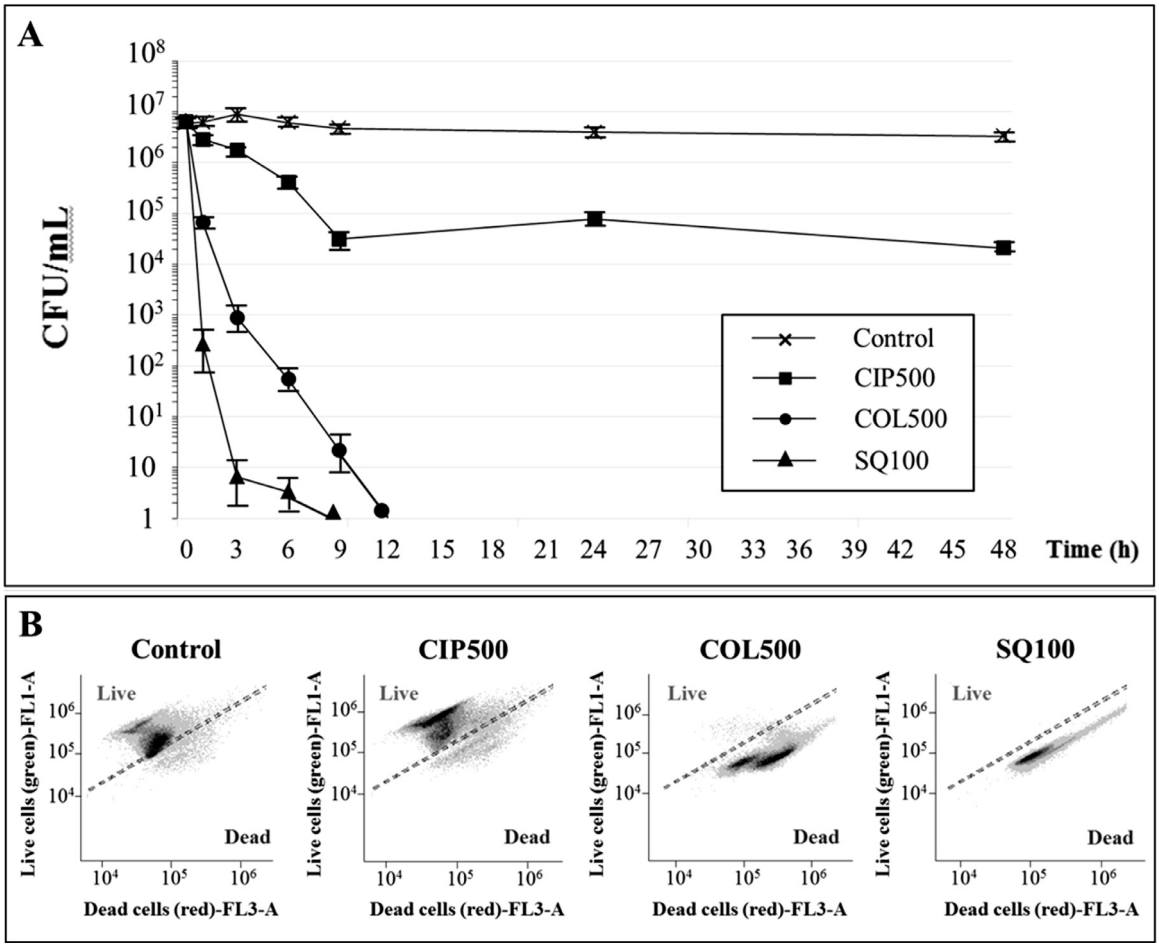

Fig. 1. Isolation of dormant populations in saline medium. (A) Culturability is given in CFU/mL depending on time (from 0 to 48 h) in the presence of ciprofloxacin at 500 mg/L (CIP500), colistin at 500 mg/L (COL500) or squalamine at 100 mg/L (SQ100) or without any compound as control. (B) Flow cytometry profiles after LIVE/DEAD® *Bac*Light™ staining. For each population (control, CIP500, COL500 and SQ100), the dot-blot represents the green (live) versus red (dead) fluorescence corresponding to fractions of cells that have, respectively, retained or not their membrane integrity after treatment. Each population (CIP500, COL500 and SQ100) is significantly different ($P < 0.001$) compared with the initial population from 3 h.

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

	LIVE/DEAD® *Bac*Light™		CTC	
	Live (%)	Dead (%)	Respiring (%)	
Isolation [a]				
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 [b]				
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	

[a] Isolated following treatment with colistin at 500 mg/L (COL500), ciprofloxacin at 500 mg/L (CIP500) or squalamine at 100 mg/L (SQ100).

[b] Cells incubated for an additional 48 h with either ciprofloxacin at 500 mg/L (CIP/CIP500), colistin at 500 mg/L (CIP/COL500 and COL/COL500) or squalamine at 100 mg/L (CIP/SQ100, COL/SQ100 and SQ/SQ100).

*** $P < 0.0001$; ** $P < 0.001$.

2.2. Effect of squalamine on dormant populations

The effect of squalamine was tested on dormant populations generated and isolated following treatment with ciprofloxacin or colistin at 500 mg/L (named CIP500 and COL500, respectively) as described in the previous section. Following 48 h of these first antibiotic treatments, cells were harvested by centrifugation at 5000 × *g* for 5 min at 4 °C. The pellet was suspended in a 1/2 volume of saline solution and the cells were incubated at 37 °C for an additional 48 h with either 500 mg/L ciprofloxacin (CIP/CIP500) to confirm dormancy or with 500 mg/L colistin (CIP/COL500), or 100 mg/L squalamine (CIP/SQ100) [14] to test their activity on dormant cells. Similarly, cells isolated after a first COL500 treatment were incubated with either 500 mg/L colistin (COL/COL500) or 100 mg/L squalamine (COL/SQ100). Finally, cells isolated after a first 100 mg/L squalamine (SQ100) were treated by an additional 100 mg/L squalamine treatment as control (SQ/SQ100). All cell densities (CFU/mL) were enumerated as described above. All experiments were performed in triplicate.

2.3. Flow cytometry and staining methods

A volume of 1 mL of each cell suspension (CIP500, COL500, SQ100, CIP/CIP500, CIP/COL500, CIP/SQ100, COL/COL500, COL/SQ100 and SQ/SQ100) was normalised at 10^6 CFU/mL and was stained in the dark using 3 µL of LIVE/DEAD® *Bac*Light™ Kit (50/50 v/v; LIVE/DEAD® *Bac*Light™ Viability/Cytotoxicity Kit; Molecular Probes-Invitrogen) for 30 min at room temperature or by 100 µL of 5-cyano-2,3-ditolyltetrazolium chloride (CTC) (*Bac*Light™ RedoxSensor™ CTC Vitality Kit; Molecular Probes-Invitrogen) at 50 mM for 2 h at 37 °C. Cells were then counted using a BD Accuri™ C6 flow cytometer (Becton Dickinson, San Jose, CA) with a low flow at 30 µL/min. Statistical analysis of bacterial populations obtained before and after treatment was performed using one-way analysis of variance (ANOVA). All quantifications were performed in triplicate.

3. Results and discussion

3.1. Isolation of dormant cells

It is generally assumed that within bacterial cultures, a stochastic population of dormant cells, including persisters, is generated [2]. In addition, dormant cells can also be induced by several environmental stresses such as starvation, oxidative stress, pH, carbon-source transitions, antibiotics etc. [3]. The persister population in *A. baumannii* cultures usually represents a small fraction of cells (<1%), similar to several bacterial species [4,7]. Thus, in order to accurately estimate the efficacy of squalamine on *A. baumannii* dormant cells, we aimed to increase the size of this subpopulation (persisters and VBNCs). For this purpose, the protocol described by Marques *et al.* was followed [13]. Three growth conditions were also tested: (i) antibiotic concentration (colistin and ciprofloxacin at concentrations ranging from 0–500 mg/L); (ii) nature of the culture medium (MHB or saline solution); and (iii) incubation time (24 h or 48 h).

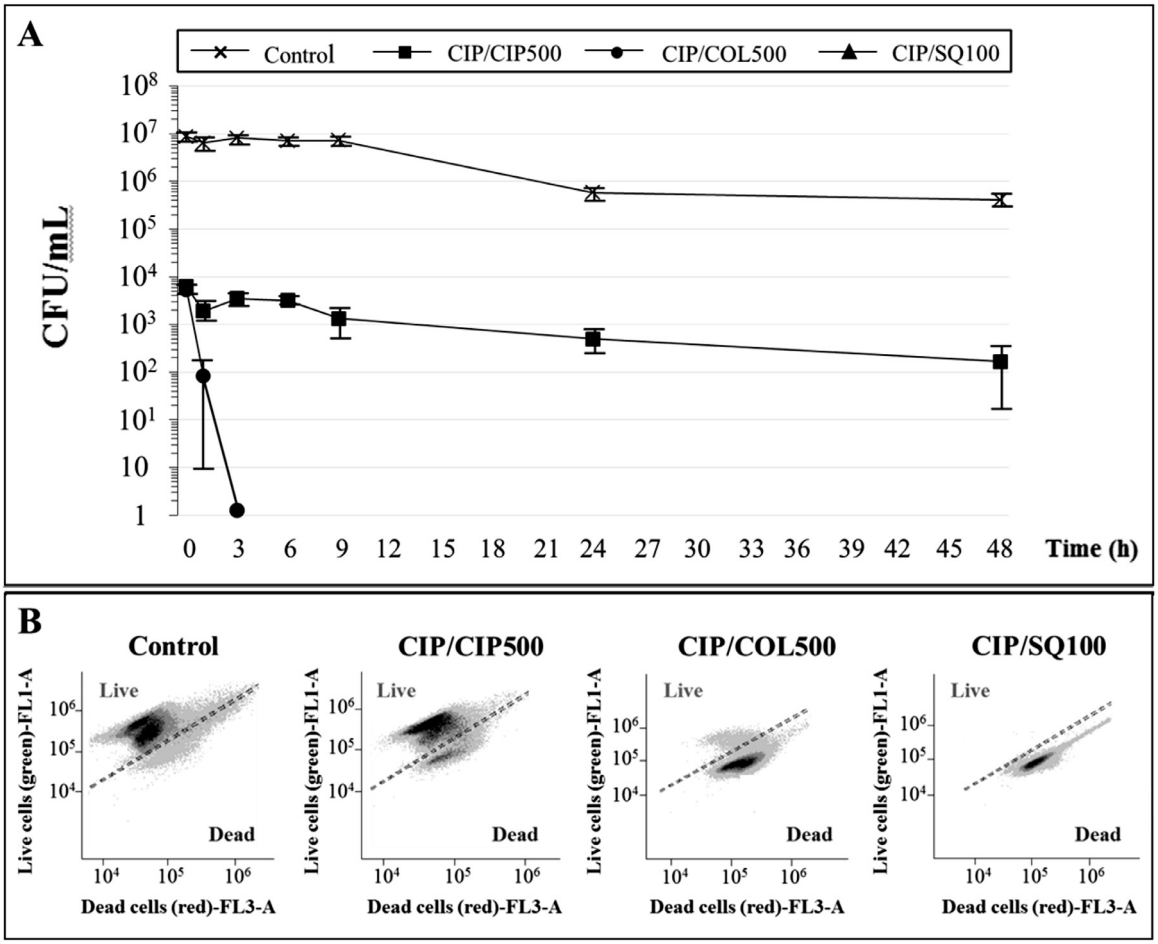

Fig. 2. Effect of squalamine on dormant populations in saline medium. (A) Culturability is given in CFU/mL depending on time (from 0 to 48 h) after a first treatment with 500 mg/L ciprofloxacin. The resulting population was treated with 500 mg/L ciprofloxacin (CIP/CIP500), 500 mg/L colistin (CIP/COL500) or 100 mg/L squalamine (CIP/SQ100) or without any antibacterial compound as control. For CIP/SQ100 cultures, the culturability limit was reached before 1 h of incubation. (B) Flow cytometry profiles after LIVE/DEAD® *Bac*Light™ staining. For each population (control, CIP/CIP500, CIP/COL500 and CIP/SQ100), the dot-blot represents the green (live) versus red (dead) fluorescence corresponding to fractions of cells that 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 with the initial population from 1 h.

Regarding colistin treatment, analysis of the culturability (proving the existence of persisters) showed that, under these stressful growth conditions, *A. baumannii* was highly tolerant to colistin at <10 mg/L (corresponding to 20 × MIC of 0.5 mg/L) whatever the culture medium used (Supplementary Fig. S1). This result was consistent with a recent study [6] pointing out the presence of a tolerant population in the presence of 10 mg/L colistin. However, this subpopulation disappeared after 24 h of treatment with increasing colistin doses of 50 mg/L or 250 mg/L in MHB and saline medium, respectively. It should be noticed that the saline medium promoted more *A. baumannii* culturability for high colistin doses than the rich medium tested.

Regarding ciprofloxacin, persisters were present at the highest concentration of 500 mg/L (i.e. 1000 × MIC of 0.5 mg/L) (Supplementary Fig. S2). The percentage of culturable/persister cells was again influenced by the nature of the culture medium, with 0.54 ± 0.41% and 0.00001 ± 0.000015% of the population after 48 h of incubation in saline medium and MHB, respectively.

Taken together, these results suggest, as already observed for *Pseudomonas aeruginosa* [13], that the persistent state in *A. baumannii* is promoted by stress, *e.g.* nutrient deprivation (saline medium) and cold shock. Although colistin treatment is often associated with a tolerance period in some ESKAPE bacteria [15], the results of the current study indicate that it does not generate a persister population, whereas this population was isolated with ciprofloxacin [13,16]. The same conclusions could be drawn for the current results on *A. baumannii*.

Thus, we next generated persisters by a 500 mg/L ciprofloxacin treatment (CIP500) in saline medium for 48 h to investigate the anti-persister activity of squalamine. This incubation time was chosen since at this time the population is the most homogeneous (Supplementary Fig. S2). The ciprofloxacin MIC of the remaining bacterial population (0.5 mg/L) was similar to the initial population MIC, thus demonstrating the presence of persisters but not resistant cells. Treatment by colistin at 500 mg/L (COL500) in the same growth conditions was used as a negative control (Supplementary Fig. S1). CIP500 and COL500 cultures were then analysed using LIVE/DEAD® *Bac*Light™ staining to evaluate the membrane integrity of the cell population and by CTC staining to explore the cell respiratory activity. Analyses of the COL500 culture, which presented no culturable cells, showed that 2.73 ± 0.52% of remaining cells retained a respiratory activity and 0.70 ± 1.15% of them retained membrane integrity. The corresponding cells may be VBNCs remaining after colistin treatment (Fig. 1B; Table 1). However, these small values have to be compared with those obtained after

an autoclaved culture and could also reflect the residual fluorescence noise (Table 1).

Regarding the CIP500 culture, while only 0.43 ± 0.11% of the initial population remained culturable (2.08 ± 0.13 × 10^5 CFU/mL) (Fig. 1), a large subpopulation, i.e. 87.73 ± 3.15%, conserved its membrane integrity and 45.83 ± 5.98% also exhibited respiratory activity (Fig. 1B; Table 1). Thus, there remained an important cell population still respiring but non-culturable. This population may be VBNCs that have not been eradicated by ciprofloxacin. As already described, high-dose antibiotic treatment can indeed induce both persister and VBNC populations, the latter being far more abundant (up to 100-fold) [17].

We can observe a higher percentage of viable cells by LIVE/DEAD staining compared with CTC. Such an overestimation of living cells in combined SYTO9/propidium iodide (PI) staining has been previously reported and may originate from PI background fluorescence as well as a higher affinity of SYTO9 for dead cells than living ones [18].

Finally, a culture grown in the same conditions treated with 100 mg/L squalamine (i.e. 50 × MIC of 2 mg/L) was performed. Squalamine did not generate persisters (Fig. 1A). No cell showed membrane integrity and only 2.73 ± 0.12% of them were still respiring. These values below those observed for autoclaved cells (Table 1) are promising for testing the efficacy of squalamine against *A. baumannii* dormant cells.

3.2. Effect of squalamine on dormant cells

Following their isolation by 48 h of ciprofloxacin treatment (Fig. 1), dormant cells were treated in the same growth conditions for an additional 48 h, either with ciprofloxacin at 500 mg/L (to confirm its inefficacy) or with colistin at 500 mg/L or squalamine at 100 mg/L [14] to evaluate the effect of these antimicrobials on dormant population (persisters and VBNCs).

The second treatment by ciprofloxacin induced a slight decrease in the number of culturable cells, similar to the reduction for the control population (CIP/CIP500) (Fig. 2). Analyses of the bacterial population by flow cytometry confirmed that viable cells remained numerous after this second treatment (18.63 ± 3.26% of the initial population still respiring) (Table 1). In contrast, the dormant population was significantly affected by colistin (CIP/COL500) and squalamine (CIP/SQ100). These two membranolytic compounds eradicated persisters in <3 h and <1 h, respectively (Fig. 2). In accordance, the percentages of cells retaining their membrane integrity, i.e. 1.90 ± 0.99% and 0.07 ± 0.03% for colistin and squalamine, respectively, or their respiratory activity (2.27 ± 1.07% and 1.77 ± 0.58%, respectively), were very low (Table 1). These data demonstrate that squalamine, like colistin, obviously kills *A. baumannii* dormant cells.

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

This study demonstrated that squalamine is able to eradicate persisters isolated following ciprofloxacin treatment. Recently, several bactericidal compounds were shown to exhibit anti-persister activity [2]. These molecules, such as SPI009 [19] and $(RW)_4$-NH_2 antimicrobial peptide [20], present a common characteristic, like squalamine [15], to induce damage to the membrane, a non-metabolically active target. This mode of action also allows squalamine to be efficient on VBNCs, as does colistin. The concomitant study both of anti-persister and anti-VBNC properties of squalamine is particularly valuable in a context of chronic infections.

4. Conclusion

Squalamine displayed great efficacy against *A. baumannii* dormant cells (*i.e.* persisters and VBNCs). Unlike colistin, the doses used here are far below the minimum haemolytic concentration (>200 mg/L) [16] and make this natural compound a very promising agent to fight against an infection relapse related to *A. baumannii* dormant cells. Further investigations are currently underway to analyse the mechanism of action involved in the anti-persister/anti-dormancy activity of squalamine.

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Competing interests

None declared.

Ethical approval

Not required.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2018.11.004.

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