Antibacterial efficacy of inhaled squalamine in a rat model of chronic 
*Pseudomonas aeruginosa* pneumonia

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**Objectives:** Squalamine is a steroid extracted from sharks with proven *in vitro* antibacterial activity. We assessed its efficacy in reducing the lung bacterial load and histological lesions when given via inhalation in a rat model of chronic *P. aeruginosa* pneumonia.

**Methods:** Sprague-Dawley rats were inoculated by tracheal intubation with 150 μL of a solution containing 10⁸ cfu/mL of agar bead-embedded *P. aeruginosa* strain PAO1. MICs of squalamine and colistin for this strain were 2–8 and 0.5–1 mg/L, respectively. Starting the day after infection, the animals were treated twice daily with aerosolized squalamine (3 mg), colistin (160 mg) or 0.9% saline for 6 days. The bacterial load and lung histological lesions were evaluated on the seventh day.

**Results:** Aerosols of squalamine and colistin resulted in a significant reduction in median (IQR) pulmonary bacterial count compared with saline [10³ (6×10²–2×10³), 10³ (9×10²–6×10³) and 10⁵ (9×10⁴–2×10⁵) cfu/lung, respectively; *P* < 0.001 for both treated groups versus saline]. The lung weight and the lung histological severity score were significantly lower in both treated groups.

**Conclusions:** In a model of chronic *P. aeruginosa* pneumonia, treatment twice daily with a squalamine aerosol for 6 days leads to a significant reduction in the pulmonary bacterial count and pneumonia lesions with an efficacy comparable to that of colistin.

**Keywords:** animal models, anti-infectious drugs, aerosols, colonization

**Introduction**

The antibiotic treatment of non-community-acquired pneumonia is a challenging issue. Among these pneumonias, pulmonary infections complicating cystic fibrosis (CF) and nosocomial pneumonia are increasingly caused by multidrug-resistant (MDR) bacteria, notably by bacteria resistant to carbapenems. The use of prolonged intravenous treatment with molecules such as colistin or aminoglycosides induces toxicity, especially renal toxicity. Consequently there is currently a great need for the development of new antimicrobial molecules that are active against MDR bacteria. In this context, innovative molecules such as aminosterol derivatives have recently gained interest due to their effective antimicrobial activity. Squalamine is a natural aminosterol extracted from *Squalus acanthias* (dogfish shark) (Figure 1). This amphiphilic steroid has shown anticancer properties in addition to antibacterial and antiviral activities. Squalamine interacts with the bacterial external membrane and destabilizes its structure, increasing the permeability of the bacteria to the external medium. Alhanout *et al.* demonstrated its bactericidal action against Gram-negative bacteria such as *P. aeruginosa* and Gram-positive bacteria such as *Staphylococcus aureus*. Squalamine also exhibited *in vitro* activity against clinical MDR strains isolated from patients with CF. In this study, the low reported MIC of squalamine for non-mucoid strains of MDR *P. aeruginosa* (2–8 mg/L) suggests its efficacy during high bacterial burden infections. Its mechanism of action is not affected by efflux pump resistance, and, to date, no bacteria has demonstrated resistance to squalamine. Squalamine has also been shown to have antifungal activity against moulds isolated from CF patients. This remarkable antimicrobial potential could be used for the treatment of nosocomial pneumonias and chronic lung infections in CF patients.
Infection with treatment

A second experiment focused on treatment efficacy. Treatments were aerosols of colistin or squalamine. The non-treated group (controls) received aerosols of saline. Since, to our knowledge, no previous published data report the use of aerosols of colistin or squalamine for pneumonia in rats, the dose of colistin was based on data in large and small animals for *P. aeruginosa* or *Acinetobacter baumannii* acute pneumonia using 8 and 6 mg/kg per administration, respectively. The dose of squalamine was selected to reach 0.15 mg/kg into lung parenchyma on the basis of a therapeutic effect at 10-fold the MIC (squalamine MIC for PAO1 = 2–8 mg/L). Previous tests on toxicity showed that 10 mg/kg of intratracheal squalamine induced no clinically and pathologically detectable toxicity (10 animals studied, data not shown). The target concentration was approximately estimated on the basis of aerosol characteristics and calculated as described below. Squalamine
was kindly provided by Professor M. Zasloff (Georgetown University, Washington, DC, USA).

Aerosols were administered using a jet nebulizer (Harvard Apparatus, Les Ulis, France) connected to an inhalation chamber containing four animals, equivalent to a ‘nose-only’ aerosol. The airflow was 6 L/min, and each aerosol administration lasted approximately 30 min. The nebulizer was charged with 5 mL of 0.9% saline alone (control group) or with saline containing either 3 mg of squalamine or 160 mg of colistin. Aerosolized solutions were buffered to obtain a pH of 7.4. The estimated amount of squalamine and colistin inhaled by the rats was calculated from the product of the concentration of the drug in the chamber, the minute ventilation of the rats (lung volume times respiratory rate) and the exposure time.23,24

Twenty-four rats infected according to our model of chronic lung infection were randomly treated with aerosolized squalamine (n = 8), colistin (Sanofi-Aventis, France, n = 8) or saline (n = 8). To evaluate the lung toxicity of the aerosol treatments, nine uninfected animals were treated for 6 days with aerosolized squalamine (n = 3), colistin (n = 3) or saline (n = 3). Aerosol treatments were performed twice per day at 12 h intervals starting the day after infection until the seventh day of evolution, totaling 6 days of treatment. This duration was decided on the basis of the lung bacterial count, which did not significantly decrease by day 7 post-infection in non-treated animals (see the Results section). On day 7, the animals were sacrificed and the lungs were removed under sterile conditions and processed for microbiological and pathological assessment.

**Histological severity score (HSS)**

Sections (3 μm thick) were obtained from the upper, mid and lower parts of the lungs, including the whole circumference. The sections were stained with haematoxylin and eosin. Examination was performed by a pathologist blinded to the group identity (H. L.). An HSS was calculated based on the number of bronchopneumonia lesions (0, no lesions; 1, <30 lesions/lung; 2, ≥30 lesions/lung; 3, confluent lesions of bronchopneumonia), as previously reported.25

**Measurement of the aerosol particle size**

Analysis of the distribution of the particle sizes was performed after the administration of an aerosol of squalamine, colistin or saline as in the animal experiments using a Malvern Mastersizer S apparatus (Malvern Instruments, UK) at a constant flow rate of 6 L/min with a 100 mm lens (measuring range from 0.5 to 175 μm). The nebulizer mouthpiece was placed at a distance of 20 mm from the lens face and 23 mm from the laser beam axis.

**Statistical methods**

Like others, this model can be characterized by variability in the extent and time course of infection, in part due to variables related to the animals. Therefore a difference of 2 logs between a treated and a non-treated group was considered relevant. Assuming such a difference, we calculated that eight animals per group were necessary to show a treatment effect, with 100% statistical power and a two-sided alpha value of 0.05. Data were expressed as the mean ± SD or the median (IQR) according to the distribution of the data. The effects of the treatments were analysed using a one-way analysis of variance or the Kruskal–Wallis test. The Student’s t-test or the Mann–Whitney rank-sum test was used for intergroup comparisons. Data analysis was performed with SPSS for Windows (Chicago, IL, USA), version 12.0. P ≤ 0.05 was considered statistically significant.

![Figure 2. Time course of lung bacterial growth during the 14 days following bacterial inoculation in untreated rats (n=5 for each timepoint). Box plots represent the median and 25th and 75th percentiles; bars represent the 5th and 95th percentiles. *P<0.05 versus baseline.](image-url)
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Effects of aerosol treatments

The size of the aerosolized particles was $3 (1.5–5.2)\mu m$ for squalamine, $2.8 (1.6–5.5)\mu m$ for colistin and $3 (1.7–5.3)\mu m$ for saline ($P=\text{not significant between drugs}$). Aerosols of saline, squalamine and colistin induced no detectable clinical or histological change in uninfected rats ($n=9$, data not shown).

At the end of the study period (7 days post-infection), all animals were alive ($n=24$). The lung bacterial count was significantly lower in animals treated with squalamine or colistin when compared with saline ($10^3 (6\times10^2–2\times10^3)$, $10^4 (9\times10^2–6\times10^4)$ and $10^5 (9\times10^4–2\times10^5)$ cfu/lung, respectively; $P<0.001$ for both treated groups versus control; $n=8$ for each group; Figure 3). No significant difference was found between the squalamine and colistin groups.

The right lung weight was also significantly lower in animals treated with squalamine or colistin than in controls ($0.84\pm0.05$, $0.98\pm0.16$ and $1.26\pm0.07$ g, respectively; $P<0.05$ for both treatment groups versus control; $n=8$ for each group; Figure 4). Pathological examination showed that the lesions of diffuse and confluent bronchopneumonia were markedly reduced in the treatment groups, and especially in the group receiving squalamine, in which areas of bronchopneumonia were rare and without abscess formation (Figure 5). The resulting HSS was lower in the squalamine- and colistin-treated groups than in the control group ($1.4\pm0.8$, $1.2\pm0.8$ and $2.7\pm0.5$, respectively; $P<0.05$ for both treatments versus control; $n=8$ for each group; Figure 6).

Discussion

The results of the present study show that 6 day regimens of inhaled treatment with 3 mg of squalamine or 160 mg of colistin induced similar reductions in both the bacterial count and histological injury in a rat model of chronic lung infection with $P.\text{aeruginosa}$. This work was the first to assess the therapeutic potential of squalamine for bacterial pneumonia.

The model of lung infection using agar beads is commonly used for experiments in vivo in the field of CF.$^{19}$ Our results from the untreated 25 rats receiving intratracheal $P.\text{aeruginosa}$ embedded in agar beads confirm the previously published reports showing a generally stable lung bacterial count up to the seventh day post-infection and 100% survival rate.$^{19,26}$ In comparison with clinical cases of hospital-acquired or ventilator-associated pneumonia, the parenchymal bacterial growth observed here, >$10^5$ cfu/mL in controls during the first 7 days, is likely to correspond to a high bacterial burden infection, since clinical cases are usually positive at $10^4$ cfu/mL as sampled using bronchoalveolar lavage.$^{27}$ The biofilm created by bacteria such as $P.\text{aeruginosa}$ prevents the diffusion of antibiotics administered intravenously, and, in CF patients, aerosols of antibiotics have been shown to improve respiratory function.$^{14}$ Treatment with new antibiotics efficient via aerosols would be a helpful therapeutic approach, increasing the therapeutic possibilities available to date.$^{14}$ Inhaled antibiotics are also increasingly used in patients with ventilator-associated pneumonia. For example, Badia et al.$^{29}$ showed that inhalation of tobramycin or imipenem resulted in high antibiotic concentrations in the lower respiratory tract without systemic toxicity. In ventilated piglets with extensive pneumonia, aerosolized amikacin or ceftazidime resulted in greater lung deposition and more extensive bactericidal effects than intravenous infusion.$^{30}$ However, ventilator-associated pneumonia and/or pneumonia caused by mucoid strains represent specific pathophysiological entities, not studied here. The efficacy of aerosol therapy and especially of squalamine would need to be evaluated in this area, e.g. using ventilated animals.

Aerosolized colistin has been particularly useful since the emergence of MDR Gram-negative bacteria. Our results in rats targeting 8 mg/kg colistin in lung agree with previous findings.
with similar doses in infected mice with carbapenem-resistant *A. baumannii*\(^{20}\) and in infected piglets with *P. aeruginosa*.\(^{22}\)

The aerosolization of squalamine and its derivatives has been shown to be feasible.\(^{38}\) Indeed, both jet nebulizers, such as that used in the present study, and vibrating mesh nebulizers produce particles of sufficiently small size to reach the distal airways.\(^{18}\)

In uninfected animals aerosolized with squalamine, we did not detect any morbidity or mortality. The histological characteristics of lungs in this group after a 6 day aerosol treatment were normal. No previous data concerning the intrapulmonary concentration of squalamine have been published yet. However, a Phase I/IIA clinical trial in oncology patients showed the safety of this molecule after a continuous intravenous 5 day infusion of 300 mg/m\(^2\)/day.\(^{31}\) Here, we did not measure the lung squalamine concentration, which represents a limitation of our study. Interestingly, according to the results of our aerosol system testing, statistical analysis demonstrated that squalamine solutions we used had aerodynamic diameters similar to those of colistin solutions. This suggests that the same proportions of aerosolized squalamine and colistin were inhaled by the animals. Otherwise, colistin and squalamine differ in their respective molecular weights, 1155 and 628 g/mol, respectively. This argues for squalamine to be effectively aerosolized and reach the lungs at least as well as colistin. In addition, squalamine, like colistin, is a positively charged molecule with a sterol core. This electro-physical property is known to favour the persistence of the molecules in the first encountered structure, here the lung. These data may favour a limited diffusion of the molecule in the blood. To determine this, we performed a test with fluorescently labelled aminosterol derivatives delivered via the tracheal route showing no blood detection of the drug.

![Figure 5. Lung histopathology 7 days after bacterial inoculation or inoculation with sterile agar beads. Haematoxylin/eosin-stained sections. Original magnification of pictures ×50. The control group (saline) section shows a diffuse bronchopneumonia with numerous and coalescent abscess formations. The squalamine group section shows rare areas of bronchopneumonia without coalescent abscess formations. Animals inoculated with sterile agar beads had normal lungs.](image)

![Figure 6. HSS (mean±SD) after 6 days of treatment with aerosols of saline (control), squalamine or colistin (n=8 for each group).](image)
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whereas fluorescence was detected in the lung. However, lung concentration was not precisely measured, but these tests argue for the absence of plasmatic diffusion (data not shown).

Conclusions
Whereas squalamine has in vitro activity against Gram-positive and Gram-negative bacteria, including MDR strains, no previous study had assessed its in vivo efficacy against infectious pneumonia. Our results suggest that squalamine administered via inhalation can reduce the lung bacterial load and bronchopneumonia-induced histological lesions with efficacy comparable to that of colistin in animals infected with P. aeruginosa embedded in agar beads. Given the need to develop new antibiotics to treat antibiotic-resistant strains of P. aeruginosa and other bacteria, squalamine could be an effective therapeutic strategy for chronic bacterial lung infection and colonization in patients. Additional studies need to be performed in order to test its efficacy in the context of mechanical ventilation. Further studies are required to better characterize its pharmacokinetics and regional diffusion into lung parenchyma, especially when using the aerosolized route.

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Transparency declarations
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