



HAL
open science

Characterization of a new aerosol antibiotic/adjuvant combination for the treatment of *P. aeruginosa* lung infections

Hana Douafer, Véronique Andrieu, Emmanuel Wafo, Jean Michel Brunel

► To cite this version:

Hana Douafer, Véronique Andrieu, Emmanuel Wafo, Jean Michel Brunel. Characterization of a new aerosol antibiotic/adjuvant combination for the treatment of *P. aeruginosa* lung infections. *International Journal of Pharmaceutics*, 2020, 586, pp.119548. 10.1016/j.ijpharm.2020.119548 . hal-03085007

HAL Id: hal-03085007

<https://hal.science/hal-03085007v1>

Submitted on 8 Jan 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Characterization of a new aerosol antibiotic/adjuvant combination for the treatment of *P. aeruginosa* lung infections

Hana Douafer¹, Véronique Andrieu², Emmanuel Wafo¹ and Jean Michel Brunel^{1*}

¹ Aix Marseille Univ, INSERM, SSA, MCT, 13385 Marseille, France. E-mail : bruneljm@yahoo.fr

²Aix Marseille Univ, IRD, APHM, MEPHI, IHU Méditerranée Infection, Faculté de Médecine et de Pharmacie, 13385 Marseille, France.

Abstract

The lack of novel classes of antibiotics as well as the constant increase of multidrug resistant bacteria are leaving the clinicians disarmed to treat bacterial infections, especially those caused by Gram-negative pathogens. Among all the investigated solutions, the design of adjuvants able to enhance antibiotics activities appears to be one of the most promising. In this context, a polyamino-isoprenyl derivative has been recently identified to be able to potentiate, at a very low concentration the activity of doxycycline against *P. aeruginosa* bacterial strains by increasing its intracellular concentration. On the other hand, since aerosol therapy allows a rapid drug administration and targets the respiratory system by avoiding the first pass effect and minimizing undesirable systemic effects, we have developed the first adjuvant/antibiotic combination in an aerosolized form and demonstrated the feasibility of such an approach. Thus, combination aerosol droplets have been demonstrated in sizes suitable for inhalation (3.4 and 4.4 μm mass median aerodynamic diameter and 54 and 60% of the aerodynamic particle size distribution less than 5 μm , as measured for the adjuvant **NV716** and doxycycline, respectively and with properties (stoichiometric 1:1 ratio of **NV716** salt to drug) that would support further development as an inhaled dosage form. Taken together, our results suggest that these molecules could be successfully delivered at the requested concentration in the lungs and then able to decrease drug consumption as well as increase treatment efficacy.

Introduction

Since their discovery, the massive and/or inappropriate use of antibiotics has led to the emergence of antimicrobial resistance.(Aslam et al., 2018; Livermore, 2004; Payne, 2008) During the last decades, we witnessed the selection of resistant bacteria whose genome has been modified favoring their development at the expense of sensitive ones. This phenomenon has resulted into a therapeutic deadlock for clinicians especially concerning Gram-negative

infections which constitute one of the major current threats to public health.(Vivas et al., 2019) Thus, *Pseudomonas aeruginosa* which is responsible for serious infections in immunocompromised patients or with cystic fibrosis lead to a high mortality rate in this population.(Bouza et al., 2002; Manfredi et al., 2000; Pang et al., 2019; Peterson and Kaur, 2018; Sharma et al., 2014; Valderrey et al., 2010) In this context, there is a need for the development of novel strategies that can modify the bacterial outer membrane integrity and/or its permeability since this latter greatly limits the diffusion of numerous hydrophilic antibiotic molecules. Recently, the concept of antibiotic adjuvants, able to restore the antibiotic activity against resistant strains was investigated. (Douafer et al., 2019) In 2019, our group reported the ability of polyamino-isoprenyl compound **NV716** to potentiate florfenicol and tetracycline activity for *Bordetella bronchiseptica* porcine pulmonary infection treatment as well as against *P. aeruginosa*, respectively. (Borselli et al., 2019; Borselli et al., 2016)

Recently, inhaled antibiotics have become a precious tool in the management of lung diseases to increase the effectiveness of the drug administration at the site of infection which allows the drug inhaled dose to be reduced compared to that administered orally or parenterally while keeping the same local effect. This considerably limits the systemic adverse effects related to the high drug dose. On the other hand, the drug local concentration in the respiratory tract is found much higher by pulmonary administration in comparison with that obtained by oral or parenteral administration.

Under these considerations, the aim of our study is to develop and evaluate a new potent pharmaceutical form intended for pulmonary administration to defeat *P. aeruginosa* lung infections, based on the use of an original antibiotic/adjuvant combination involving doxycycline and polyaminoisoprenyl derivative **NV716**.

Materials

Bacterial strains

The bacterial strains used in our study consist in the *Pseudomonas aeruginosa* reference strain PAO1 as well as clinical isolates collected from Laveran Hospital (Marseille). The strains were stored in 15% (v/v) glycerol at -80 °C for cryo-protection.

Antibiotic

The antibiotic used in this study is doxycycline which was purchased from Sigma -Aldrich (Saint Quentin Fallavier, France) Doxycycline was dissolved in sterile distilled water and the stock solution is prepared at a concentration of 6.4 mg/mL.

Nebulizer systems

Three different nebulization systems were used for the nebulization of the different solutions in all the tests carried out. The pneumatic air-jet nebuliser PARI LC Plus® has been selected for the aerodynamic characterization of the different aerosols under various experimental conditions. The PARI LC Plus® is connected to the PARI BOY® SX compressor which generates compressed air at an operating pressure (connected nebulizer) of 1.6 bar. Two other nebulizers were used to study the effect of the nebulizing device on the aerodynamic properties of aerosols generated from our formulation: the PARI Sprint® (air-jet nebuliser) coupled to the PARI BOY® SX compressor and the PARI eFlow® rapid: a vibrating-mesh nebulizer which operates by vibration of a sieve in contact with the liquid to be sprayed. The three nebulizing devices were provided by the PARI PulmoMed SARL, Fr company.

Methods

General procedure for the synthesis of polyaminoisoprenyl derivative NV716

To a solution of spermine (450 mg, 2.27 mmol) and triethylamine (450 μ L, 4.5 mmol) in distilled THF (10 mL) is added dropwise farnesyl chloride (480 mg, 2 mmol) in distilled THF (15 mL). The reaction mixture is stirred at room temperature for 24 h. and evaporated to dryness. The crude residue is purified by column chromatography (eluent CH₂Cl₂/MeOH/conc. NH₄OH, 7:3:1) to afford the pure desired compound as a yellow solid in 64% yield. ¹H NMR (MeOD, 250 MHz): δ = 5.05-4.93 (m, 3H), 2.93-2.57 (m, 14H), 2.19-1.92 (m, 10H), 1.63-1.87 (m, 23H). ¹³C (MeOD): δ = 142.24, 134.83, 131.09, 124.86, 124.17, 117.32, 47.90, 47.69, 47.64, 46.61, 44.01, 40.60, 39.83, 33.97, 31.20, 26.97, 26.30, 25.66, 25.11, 24.90, 17.62, 16.90, 15.93. C₂₅H₅₀N₄ MS (ESI+) m/z 407.41 (100%, [M + H]⁺).

The polyaminoisoprenyl derivative obtained is converted into its corresponding hydrochloride salt by adding anhydrous HCl methanol solution (2M) (6 equivalents) in anhydrous methanol with stirring maintained at room temperature for 16 h. Methanol is evaporated until residual volume of about 50 mL is reached. Diethyl ether is added (about 100 mL) and the resulting precipitate is filtered off to give the pure desired salt as a white solid.

Antibiotic susceptibility testing.

The antibacterial activity of doxycycline and adjuvant **NV716** was evaluated by determining their Minimum Inhibitory Concentration (MIC) against various strains of *P. aeruginosa*. The MIC is defined as the lowest concentration of a compound required to induce a visible inhibition of the bacterial growth after an incubation time of 24 h at 37°C. The MICs of the two

compounds against the different strains were established by the broth microdilution method according to the recommendations of the French Society of Microbiology Antibiogram Committee (CA-SFM). (Wikler, 2006) From the stock solutions of doxycycline and **NV716**, serial dilutions (from 128 µg/mL to 0.25 µg/mL for doxycycline and from 200 µg/mL to 0.39 µg/mL for **NV716**) were realized from the 3rd line of the microplate, the first being reserved to the negative control (culture broth alone) and the second to the positive one (bacterial suspension alone without any antibacterial compounds). The MIC was determined with an inoculum of 10⁵ CFU in 200 µL of MHII broth containing twofold serial dilutions after incubation for 18 hours at 37 °C. The experiments were performed in triplicate.

Determination of the MIC of doxycycline in the presence of NV716

The antimicrobial activity of the doxycycline/compound **NV716** combination was evaluated in two ways against a large panel of *P. aeruginosa* strains. In a first step we evaluated the influence of derivative **NV716** used at a 10 µg/mL concentration on the MIC of doxycycline. Thus, a two-fold serial dilution of doxycycline (from 128 µg/mL to 0.25 µg/mL) was performed from the starting solution (6.4 mg/mL). In a second time, doxycycline concentration in each well was fixed at 2 µg/mL which corresponds to the doxycycline susceptibility threshold according to the CLSI (Clinical and Laboratory Standards Institute), in order to determine the lowest concentration of adjuvant capable of decreasing the MIC of doxycycline at the sensitivity threshold.

For both tests, the bacterial suspensions were prepared from colonies grown overnight. The bacterial concentrations were adjusted to 1.5×10^5 CFU/well. The MIC of doxycycline / **NV716** combination was determined after 18 h of incubation at 37 °C. The test was repeated three times independently.

Aerodynamic characterization of aerosols using the Next Generation Impactor.

The aerodynamic characterization of the different inhaled solutions was carried out according to the recommendations of the European Pharmacopoeia (Ph. Eur.), using the Next Generation Impactor (NGI) [2.9.18 monograph]. The NGI is a high-performance, precision and particle classifying impactor, composed of seven-stages plus a micro-orifice collector (MOC). A USP throat was attached at the entry of the impactor and a mouthpiece adapter was used at the NGI induction port to facilitate the assembly of the nebulizer to the USP throat. The NGI was operated at a rate of 15 L/min in accordance with the pharmacopoeial recommendations for the evaluation of inhaled solutions [2.9.44 monograph]., the flow rate was measured by a Copley

flowmeter (Copley Scientific, UK), a flow regulator (Copley Scientific, UK) and a pump (Copley Scientific, UK) were assembled at NGI (Figure 1). An internal filter was required and placed before the last MOC (Micro-Orifice Collector) stage to collect all extra-fine droplets at the output of the NGI. The NGI was cooled for at least 90 min at 5°C to reduce droplet evaporation, limit the possibility of particle size change, and provide an environment close to 100% relative humidity inside the NGI. The nebulization must begin in less than five minutes after removing the NGI from the refrigerator.

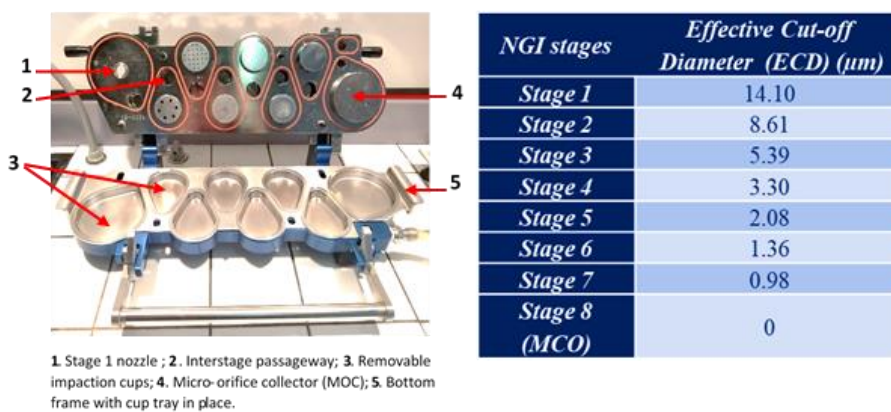


Figure 1. The next generation impactor (NGI) used for aerosols aerodynamic characterization. The nebulizer is attached to the USP throat, 5 mL of the inhaled solution was placed in the nebulizer reservoir and the nebulization was performed for the time determined by the test. At the end of the nebulization the pump is switched off and the amount of active substance deposited on each stage of the NGI impactor as well as on the USP throat, the internal filter and the nebulizer mouthpiece is recovered with a sufficient volume of ultra pure water. The amount deposited on each stage is determined by Gas chromatography coupled to mass spectrometry assay. All the experiments were performed in triplicate.

Gas chromatography–mass spectrometry GC–MS conditions.

The chromatographic sample separation and analysis were performed using an Agilent Technologies 6890N Network GC System (Agilent 7683B Injector and autosampler) and coupled to Agilent 5973 Network mass spectrometer detector. The separation was achieved in a 30m \times 0.25mm internal diameter (I.D.) \times 0.25 μm film Thickness, Zebron ZB50 capillary column (Phenomenex). The injection volume was 1 μL , and the helium carrier gas flow was 1mL min⁻¹. The temperatures of the MS transfer line, ion source, and quadrupole were set at

280, 230, and 150°C, respectively. The injector temperature was 250°C (splitless), and the oven was programmed from 40 (hold time: 0.5 min), to 280°C at 10° C min⁻¹, (hold time: 5 min). Selected ions for detection for each compound: **Doxycycline**: 393; 315; 255; 175;147; **NV716**: 205; 177; 159, 137.

Standard solutions preparation for GC-MS analysis.

Two concentrated stock solutions of each of the two compounds (doxycycline and **NV716**) were prepared by dissolving sufficient quantities (1 mg of each compound separately) in ultrapure water to obtain a concentrated solution (1000 µg/mL) which serves as a stock solution for the preparation of diluted solutions for calibration purposes. Solutions of decreasing concentrations (500, 250, 125, 62.5 and 31.25 µg/mL) for each compound (doxycycline and **NV716**) were prepared by two-fold dilution series. The solutions were stirred for 3 minutes and then transferred to vials before being directly subjected to the mass-coupled gas chromatographic assay.

Aerodynamic characterization of the aerosols generated

The inhalation solution of each compound was evaluated and then the solution of the two compounds taken together (doxycycline and **NV716**) was tested (Table 1). The nebulizer used in this part of the study is the PARI LC Plus® and 5 mL of each solution were added to the nebulizer reservoir for each test. The concentration of every drug in the solution is 10 mg/mL and has been chosen according to the limit of detection of the two compounds by our GC-MS apparatus on the different stages of the impactor. The duration of the nebulization was fixed at 6 min. All the experiments were performed in triplicate. (S1)

Determination of the influence of the active substances' concentrations and duration of nebulization on the measured aerodynamic properties of a combination aerosol

First, the aerodynamic properties of the aerosol generated from the solution of the two compounds taken together (doxycycline and **NV716**) at different concentrations were evaluated. The duration time of nebulization was set at 6 minutes and four different concentrations were used for each compound: 10, 15, 20 and 30 mg/mL. (S2)

The influence of the nebulization time (1, 3, 6 and 10 min) on the aerodynamic properties of the considered aerosol was evaluated whereas the concentration for each compound was fixed at 10 mg/mL (Table 2). The nebulizer used in this two-test series is the PARI LC Plus® nebulizer and the reservoir is filled with 5 mL of the inhalation solution. (S3)

Data analysis

The calculation of the different parameters required for the aerosol aerodynamic characterization was performed by using the Copley Inhaler Testing Data Analysis Software, (CITDAS, Copley Scientific, UK). The Effective Cut-off Diameter (ECD) of each stage of the Next Generation Impactor (NGI) varies with the flow rate used. For a flow rate of 15 L/min as used in our study the default effective cutting diameters of the different stages are mentioned in Figure 1.

The Fine Particles Fraction (FPF) corresponds to the fraction of particles whose aerodynamic diameter is less than 5 μm and is calculated as follow:

$$\text{FPF (\%)} = \frac{\text{Fine Particles Dose (diameter <5 } \mu\text{m)}}{\text{Delivered Dose}} * 100$$

The Delivered Dose (DD) represents the total mass of drug administered into the device (NGI) from the mouthpiece of the inhaler excluding the amount deposited on the nebulizer.

The Mass Median Aerodynamic Diameter (MMAD) obtained by interpolating the aerodynamic diameter corresponding to 50% of particles undersize (or Probit 5) and the Geometric Standard Deviation (GSD) are calculated from the graph representing the cumulative fraction of the active substance relative to the effective cut-off diameter on log-probability paper (the distribution being assumed to be lognormal) (S4).

Results

Derivative **NV716** was prepared according to the following synthetic pathway (Figure 2). This strategy involved a direct nucleophilic substitution of spermine on farnesyl chloride leading to the formation of the expected compound in 64% yield that was subsequently transformed in its hydrochloride soluble salt.

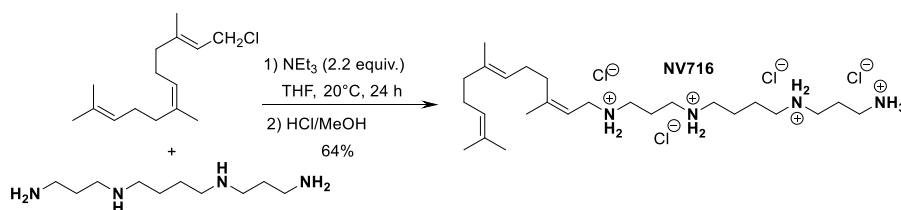


Figure 2. Synthetic pathway for the adjuvant **NV716**

As illustrated in Figure 3, compound **NV716** was successfully assayed for its ability to potentiate doxycycline activity against 64 *P. aeruginosa* bacterial strains.

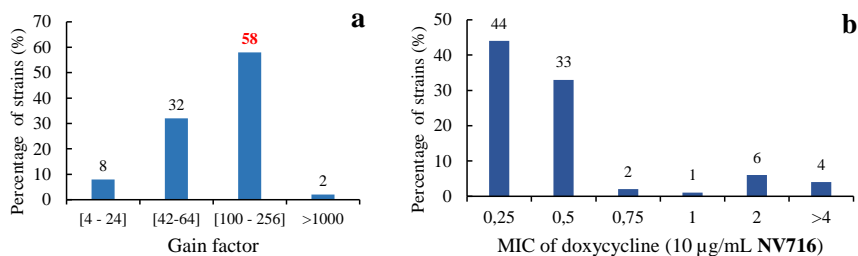


Figure 3. Percentage of *P. aeruginosa* strains (a) and gain factor encountered (b) related to the restoration of doxycycline activity in the presence of adjuvant NV716

The results clearly indicate a potentiation of the doxycycline activity in presence of NV716, which induced a decrease in the MIC of doxycycline from 64 µg/mL to 0.25-0.5 µg/mL with a gain factor of 42-256-fold when used at 10 µg/mL concentration. The MIC of more than 96% of the considered *P. aeruginosa* bacterial strains have been lowered to a level less than or equal to the sensitivity threshold of *P. aeruginosa* (2µg/mL).

The aerodynamic properties of the aerosol for each compound, doxycycline and NV716, are summarized in Table 1. Thus, the two compounds nebulized separately using the PARI LC Plus® nebulizer presented a Mass Median Aerodynamic Diameters MMADs of 3.47 and 4.54 µm and a percentage of FPF of 55 and 57%, for doxycycline and NV716 respectively.

<i>Nebulizer</i>	<i>Active substance</i>	<i>Concentration (mg/mL)</i>	<i>Nebulization duration (min)</i>	<i>Flow (mL/min)</i>	<i>MMAD (µm)</i>	<i>GSD</i>	<i>FPF (%)</i>
PARI LC Plus®	Doxycycline	10	6	0.25	3.47	3.24	55
	NV716	10	6	0.38	4.54	1.80	57

Table 1. Aerodynamic parameters for aerosols involving separately doxycycline and NV716.

On the other hand, the mass of each compound deposited on each stage of the impactor, the throat and the nebulizer mouthpiece, resulting from the nebulization of each compound alone by the PARI LC Plus® nebulizer has been determined as illustrated in Figure 4.

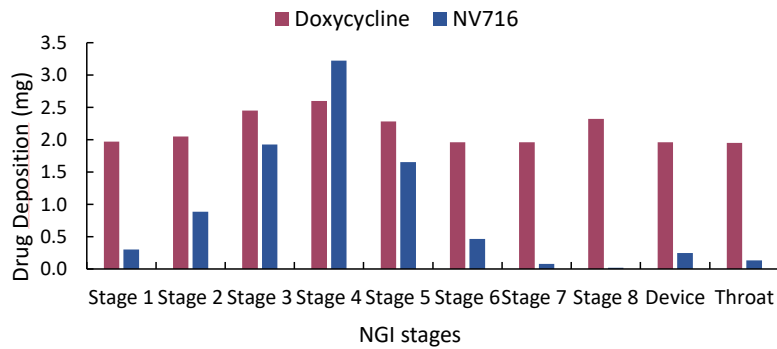


Figure 4. Mass distribution of doxycycline and NV716 aerosols on the NGI stages.

The *in vitro* mass deposition histogram of the two compounds on the NGI stages shows a Gaussian distribution with a maximum of deposition at the stage 4 of the impactor and this for the two aerosols tested.

The parameters of nebulization of a doxycycline/NV716 combination solution (nebulized volume and nebulization flow) obtained with the PARI LC Plus® nebulizer (in triplicate) as well as the conditions used (concentration of each compound and duration of nebulization) are summarized in **Table 2**.

Assay	Nebulized volume (ml)	Flow (mL/min)
1 st	2.06	0.34
2 nd	1.87	0.31
3 rd	1.72	0.29

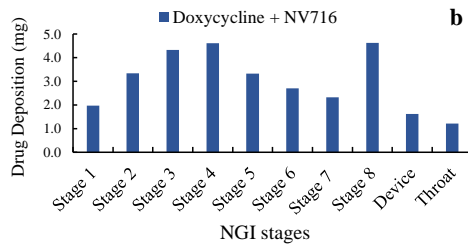


Table 2. Nebulization parameters (a) and drug mass deposition (b) of the doxycycline/NV716 combination aerosol generated by using PARI LC Plus®

Thus, the nebulisation for 6 min allows nebulization of volumes varying from 1.72 to 2.06 mL with nebulization flow rates ranging from 0.29 to 0.34 mL/min. The aerodynamic properties of the combination aerosol droplets as well as the percentage of fine particles obtained in triplicate are summarized in Figure 5. It is noteworthy that the two compounds nebulized together (doxycycline and NV716) presented a Mass Median Aerodynamic Diameter of droplets ranging

from 3.06 to 4.16 μm with a geometric standard deviation varying from 2.60 to 3.09 and a fine particle fraction FPF from 55 to 64%.

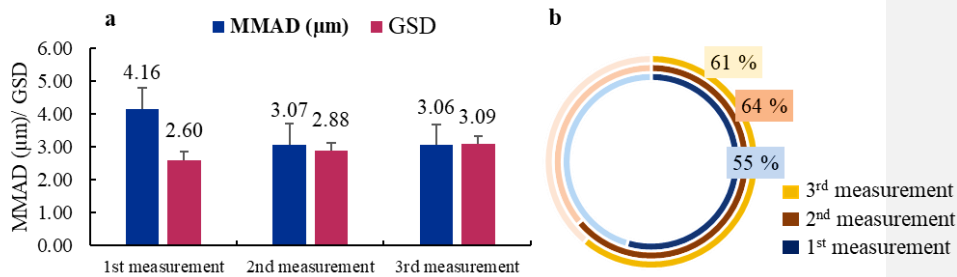


Figure 5. Droplets characterisation (a) and Fine Particles Fraction FPF (b) obtained from the doxycycline /NV716 combination nebulization

The Mass Median Aerodynamic Diameters MMADs as well as the Fine Particles Fraction FPF obtained from the nebulization of the two compounds together (doxycycline and NV716) using the PARI LC Plus® nebulizer according to different concentrations of the two compounds involved as well as different nebulization times are summarized in Figure 6.

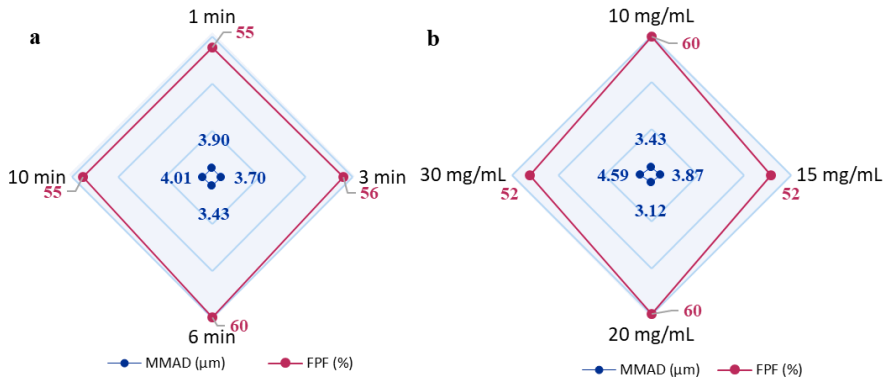


Figure 6. The combination aerosol aerodynamic properties according to different doxycycline/NV716 concentrations (a) and different durations of nebulization (b)

The Mass Median Aerodynamic Diameters MMADs of the combination aerosols obtained by varying the concentration of the two active substances ranged from 3.12 to 4.59 μm , and 3.43 to 4.01 μm depending on the nebulization duration time, respectively. Furthermore, the fine particles fraction (FPF) obtained by modifying the concentration of the two compounds

Commenté [AV1]: Ce n'est pas l'inverse ? durations of nebulisation (a) & concentrations (b).

involved ranges from 52 to 59% and 55 to 59% by varying the duration of nebulization, respectively.

Additionally, a study dealing with the influence of the nebulizer device on the aerodynamic properties of the aerosol generated from the combination solution was performed using three different nebulizers (PARI LC Plus®, PARI Sprint® and PARI eFlow®). The conditions of nebulization (concentration of the two-active substances, duration of nebulization and nature of the nebulization device) as well as the nebulization parameters (nebulized volume and nebulization rate) obtained with each nebulizer are summarized in Table 3. It clearly appears that the PARI LC Plus® nebulizer allowed the nebulization of 1.8 mL of the aerosolized solution in 6 min with an average flow rate of 0.31 (\pm 0.04) mL/min. The PARI Sprint® nebulizes at an average flow rate of 0.60 (\pm 0.02) mL/min 3.5 mL of the tested combination during a 6 min period. On the other hand, under the same duration time of nebulization the PARI eFlow® nebulizer was able to nebulize 2.9 mL with an average flow rate of 0.45 (\pm 0.15) mL/min.

<i>Nebulizer</i>	<i>Doxycycline (NV716) (mg/mL)</i>	<i>Nebulized volume (mL)</i>	<i>Flow (mL/min)</i>	<i>MMAD (μm)</i>	<i>GSD</i>	<i>FPF (%)</i>
<i>PARI LC Plus®</i>	10 (10)	1.88	0.31	3.43	2.86	60
<i>PARI LC Sprint®</i>	10 (10)	3.54	0.59	4.42	2.31	54
<i>PARI eFlow®</i>	10 (10)	2.90	0.45	4.23	1.85	60

Table 3. Nebulization parameters and aerodynamic properties of the combination aerosol obtained by using three different nebulizers

Figures 7a and 7b summarize the aerodynamic characterization parameters (MMAD, GSD and FPF) of the aerosols produced by the PARI LC Plus®, the PARI Sprint® and the PARI eFlow® nebulizers for the antibiotic-adjuvant combination (doxycycline and NV716). Thus, the aerosol produced by the PARI Sprint® had a MMAD of 4.40 μ m and a GSD of 2.8 whereas nebulization with the PARI LC Plus® lead to an aerosol with a MMAD of 3.40 μ m and a GSD of 2.3. The aerosol droplets produced by the PARI eFlow® vibrating mesh nebulizer had an MMAD of 3.4 μ m and a GSD of 1.8. The fine particles fractions FPFs ranged from 60% for the PARI LC Plus® device, 54% for the PARI Sprint® and to 60% for the PARI eFlow®.

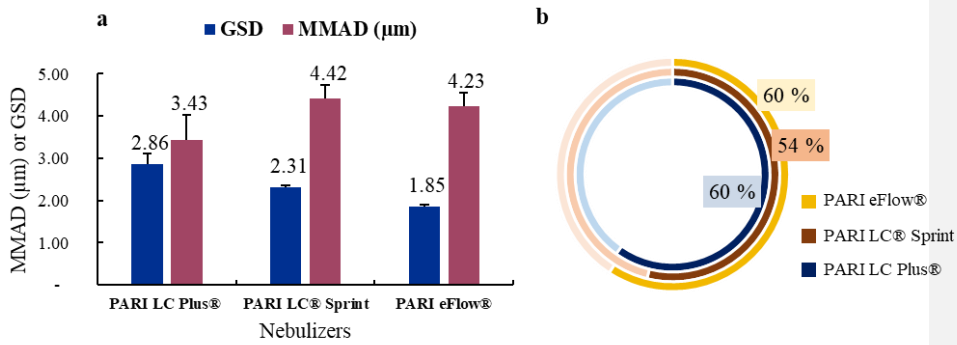


Figure 7. The combination aerosol droplets characterization and Fine Particles Fraction obtained by using three different nebulizers

The histograms of the two compounds mass distribution (doxycycline and **NV716**) on the different stages of the impactor, the throat and the nebulizer mouthpiece obtained with each nebulizer: the PARI LC Plus®, the PARI Sprint® and the PARI eFlow® are presented in the Figure 8. The three nebulizers gave aerosols with maximum of deposition on stage 4 of the impactor. The three distribution histograms obtained by changing the nebulizer device had a Gaussian appearance.

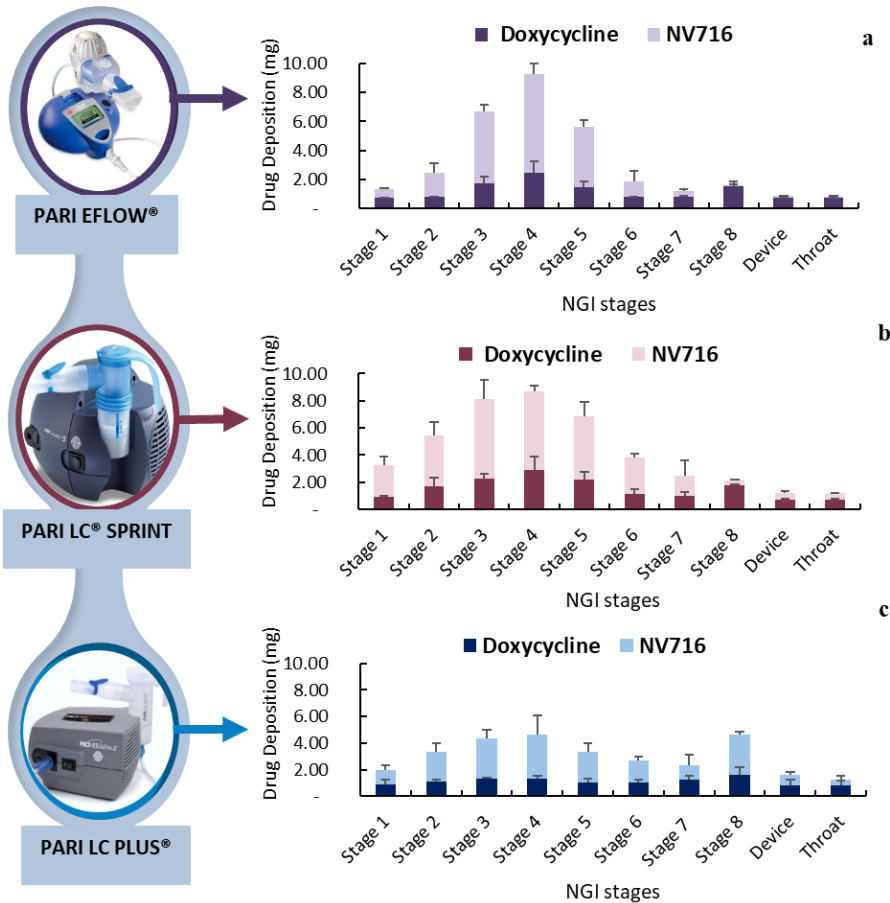
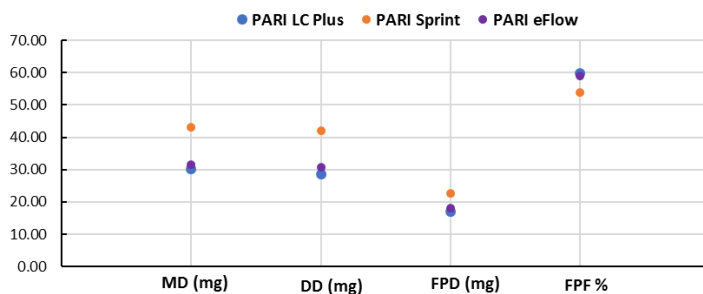


Figure 8. Mass distribution of doxycycline and **NV716** on the NGI stage obtained by using three different nebulizers

Additionally, a comparative study of the performance of the three nebulizers clearly underline different specificities as illustrated in Figure 9. The results clearly indicates that although the PARI sprint nebulizer gives a slightly lower FPF (54 %) than those obtained with the PARI LC Plus (60%) and the PARI eFlow® (60%), it gives the highest nebulized dose of 43 mg compared to 30 and 31 mg nebulized with the PARI LC Plus and PARI eFlow®, respectively. It is noteworthy that these results are due to its high performance allowing the nebulization of a volume superior to those nebulized by the two other nebulizers. Consequently, the delivered dose is 42 mg compared to 28 and 30 mg delivered by the PARI LC Plus and the PARI eFlow®, respectively.



Nebulizer	Metered Dose MD (mg)	Delivered Dose DD (mg)	Fine Particle Dose FPD (mg)	Fine Particle Fraction FPF (%)
<i>Pari LC Plus</i>	30.14	28.51	16.89	60
<i>Pari sprint</i>	43.21	42.02	22.66	54
<i>Pari efflow</i>	31.51	30.67	18.12	60

Delivered Dose : corresponds to the total mass of drug delivered into the apparatus.

Fine Particle Dose : corresponds to the dose of particles (mg) with an aerodynamic diameter less than 5 μm .

Figure 9. Comparison of the performance of the three nebulizers (PARI LC Plus, PARI Sprint and PARI eFlow®).

Discussion

In the search for the development of new potent therapeutic agents, aerosol therapy appears as one of the most interesting way for adjuvant-antibiotic combinations administration at the therapeutic dose. It offers the advantages of effectively targeting the respiratory tract with fast obtaining of the pharmacological effect while avoiding the effect of the first pass effect and minimizing undesirable systemic side-effects.(Lemarié and Diot, 2005) On the other hand, by this means the antibiotic and the adjuvant can be delivered simultaneously near the site of infection and at the active concentration required. Because of these benefits, aerosol therapy has expanded strongly in the recent years and the lung has become a gateway for systemic drugs and not just an organ to treat.(Reychler et al., 2007)

Inhalation is a very commonly used technique for administering medication to patients to treat a variety of lung diseases.(Le Brun et al., 2000; Newman and Clarke, 1983) The liquid atomization (or nebulization) is the process allowing the conversion of a liquid form in fine droplets using a particular device: the nebulizer.(Lange and Finlay, 2006) Nevertheless, the complexity of the pulmonary route in drugs administration stems from the need to convert drug formulations into fixed dose aerosol clouds as quickly as possible with optimal properties, and then delivers them in a short time.

Nebulizers are used to generate aerosols from solutions and sometimes suspensions of drugs for inhalation. Thus, with the right device and formulation, a wide range of doses of many drugs can be nebulized.(O'Callaghan and Barry, 1997) However, the effective use of nebulizers requires a good understanding of how they work and the factors influencing their performance.(Le Brun et al., 2000) They are generally used in situations of severe airway obstruction where insufficient patient coordination does not allow the use of other systems such as Dry Powder Inhalers (DPIs) or Metered Dose Inhalers (MDIs). For example, they are particularly recommended for young patients who cannot manage other devices.(Le Brun et al., 2000) There are three types of nebulizers: pneumatic (air jet) nebulizers, ultrasonic nebulizers and vibrating membrane nebulizers..)Dessanges, 1998(However, the most commonly used type of nebulizer is the jet nebulizer, which uses a source of compressed gas (compressor) to create the aerosol from the drug solution.(O'Callaghan and Barry, 1997) The compressed gas is forced through a tube system connected to a nozzle. As the cross section of the tube decreases, the air velocity increases creating a low-pressure zone around the nozzle (Venturi effect). The pressure drop created because of the jet passing high velocity through the Venturi nozzle causes the liquid formulation to rise on a feed tube from the liquid reservoir (Bernoulli effect). Thus, a complex process of fragmentation of the liquid takes place allowing the creation of aerosol droplets. The large droplets can then impinge on the walls of the nebulizer, recycling into the tank. However, sufficiently small droplets bypass these barriers (secondary droplets) and form the breathable aerosol generated by jet nebulizers.(Le Brun et al., 2000) Therefore, the design and dimensions of nebulizers largely influence on the characteristics of the aerosol. This reason reinforces the idea that nebulizers should be evaluated as a multi-component system for the generation of respirable aerosols. (Carvalho and McConville, 2016; McCallion et al., 1996; Mercer, 1981) In the case of ultrasonic nebulizers, the drug solution is nebulized by the energy of high-frequency sound waves (cavitation effects). They can be equipped with nebulization power adjustment. (Dautzenberg et al., 2007) Vibrating mesh nebulizers are micro-pump systems. This technology is the result of energy that forces the liquid through the small openings in a plate or membrane. (Carvalho and McConville, 2016)

In our case, the choice of the pneumatic (air jet) nebulizers was based on the fact that they are the most commonly used drug delivery device for pulmonary diseases (Rangaraj et al., 2019) and on their adaptability to the nebulization of medicines mixture. First, we started our study by using the PARI LC Plus® device, which is the most commonly used pneumatic nebulizer in the field of nebulization. Then the Pari company proposed us the PARI Sprint®, which represents the new version of its pneumatic nebulizers. Thus, the use of these two pneumatic

nebulizers allowed us to compare the performance of an old and a new pneumatic nebulizer. Furthermore, to compare their efficiency with respect to another technology, a vibrating membrane nebulizer (the PARI eFlow®) which overcomes the disadvantages of both jet and ultrasonic nebulizers.(Chan et al., 2014) was involved.

Finally, ultrasonic nebulizers have not been used since they are already less and less used for pulmonary drug delivery. Indeed, they lead to the generation of droplets with relatively large diameters (3.7- 10.5 μm) compared to that encountered for pneumatic nebulizers (1.2- 6.9 μm),(Groneberg et al., 2003) which does not fit with the active ingredients deposition in the part of the lungs targeted by our formulation.(Radan, 2017)

The novelty of the work described herein consists of (a) the use of an adjuvant-antibiotic combination for *Pseudomonas aeruginosa* infection treatment; (b) its preparation as a water soluble form for aerosolization and (c) preparation of the salt as a aerosolized dosage form to capitalize on local delivery to the lungs. In this context, synthesis of **NV716** was easily performed in high chemical yield as a water-soluble salt. Its use in combination with *P. aeruginosa* strains. Thus, the doxycycline / **NV716** combination was more bactericidal than the pure compounds taken alone which supports the hypothesis that the combination form increases the killing effect against more than 92% of the strains presenting a gain factor with respect the doxycycline up to 42 (Figure 3). Moreover, 77% of the strains are sensitive towards the doxycycline/**NV716** combination and involving a MIC for doxycycline varying from 0.25 to 0.5 $\mu\text{g}/\text{mL}$ in the presence of **NV716** at a 10 $\mu\text{g}/\text{mL}$ concentration suggesting the potent use of such a combination for aerosol therapy.

In this context, an aerosol with optimal properties should be composed of particles neither too small (to avoid their elimination during the exhalation) nor too large (to avoid their deposit in the upper ways, the mouth and the throat).(Patton and Byron, 2007) A better understanding of the various factors influencing the inhaled particles deposition increases the precision of lung deposition and allows to target a specific disease and reduce the exposure of normal respiratory tract to drugs. (Cheng, 2014) (Ariyananda et al., 1996)(Ferron, 1994)(Thompson, 1998) Thus, it is important to determine the particle size distribution of droplets generated by the inhalation device used as well as the percentage of particles of therapeutic interest delivered by inhalation. The main parameter used to describe and characterize the inhaled particles is the aerodynamic diameter 'Dae' which by definition is "the diameter of a sphere having the same speed of fall as the particle and an equal density of 1 g/cm^3 .(Ariyananda et al., 1996; Lemarié and Diot, 2005) The statistical interpretation of the aerosol particles size distribution is related to the Mass Median Aerodynamic Diameter (MMAD), that divides the mass of particles in two halves

equally distributed on both sides of the aerosol MMAD.(Lemarié and Diot, 2005) Thus, the MMAD helps predict the behaviour of inhaled aerosol particles, including their likely deposition sites.

There are three mechanisms by which inhaled particles are deposited within the respiratory tract: impaction, sedimentation and diffusion.(Dessanges, 1998) Due to their relatively high mass, the larger particles are strongly affected by velocity, and therefore settle by inertial impaction in the upper respiratory tract. However, smaller particles tend to reach the deep lungs where they are deposited by sedimentation. At the smaller end of the scale, the finer particles undergo Brownian motion and are generally eliminated with exhaled air. According to the recommendations for good aerosol therapy practices, the deposition site at the respiratory system level is defined according to the size of the inhaled particles, particles having an aerodynamic diameter greater than 5 µm are deposited by impaction in the Oro-Rhino-Laryngological sphere (ORL), particles with a size between 2 and 6 µm migrate to the bronchi, particles with an aerodynamic diameter between 0.5 and 3 µm can reach the deep lungs and particles with an aerodynamic diameter between 0.6 and 0.3 µm will be removed during the exhalation (Dautzenberg et al., 1998). Overall, particles with an aerodynamic size between 1 and 5 µm can be deposited in the deep lungs.(Carvalho and McConville, 2016; Carvalho et al., 2011)

Thus, the aerodynamic characterization of the aerosols of each one of the two compounds (doxycycline and **NV716**) showed mass median aerodynamic diameters MMADs less than 5 µm (3.47 and 4.54 µm) in accordance with the standard recommendations for therapeutic aerosols. In addition to the MMAD, the aerodynamic characterization of aerosols is also done by the Fine Particles Fraction (FPF) which represents "the percentage of particles having an aerodynamic diameter (D_{ae}) between 1 and 5 µm, size adapted to a pulmonary deposition.

In this context, the two aerosols presented more than 55% of FPF meaning that more than 55% of the particles of each aerosol have an aerodynamic diameter less than 5 µm, allowing them to reach deep regions of the lungs (site of infection) in therapeutically sufficient amounts. Furthermore, the *in vitro* aerosol deposition profiles on the various stages of the NGI showed a similarity of the dispersion of the two compounds nebulized separately, with a maximum of deposition on the stage 4 of the impactor which represents the secondary bronchi. The geometric standard deviation GSD obtained with the doxycycline aerosol of 3.24 is greater than that obtained with the **NV716** aerosol, resulting in a wide dispersion of the aerodynamic diameter of doxycycline droplets around the mean (MMAD) compared to the dispersion of the aerodynamic diameters of the **NV716** droplets. This results in a larger amount of doxycycline

deposited on the 1st and 7th stages compared to **NV716** very minimal on these two stages. Moreover, the results of aerodynamic characterization of the antibiotic/adjuvant combination aerosol (doxycycline/**NV716**) in triplicate showed that simultaneous nebulization of the two compounds generates particles with mass median aerodynamic diameters MMADs suitable for pulmonary administration and consistent with recommendations for therapeutic aerosols MMAD <5 µm. Thus, the geometric standard deviation GSD of the combination aerosol droplets diameter was found to range from 2.06 to 3.06 which gives a wide dispersion of the aerodynamic diameter of the inhaled particles around the mean MMAD allowing them to be dispersed at the different stages of the impactor and so to cover the whole respiratory system. The *in vitro* distribution profiles of the combination aerosol on the different stages of the NGI, the throat and the nebulizer mouthpiece confirm this wide dispersion of the particles and present a good Gaussian distribution of the two compounds with maximum deposition at stage 4 of the impactor which represents the secondary bronchi, the same maximum deposition having been obtained for each compound nebulized alone. Finally, it is noteworthy that the simultaneous nebulization of the two compounds permitted to obtain up to 63% of FPF ie 63% of the combination aerosol droplets have a therapeutic interest and can reach the site of the infection, which shows a better efficiency of the aerosol of the combination compared to those of each compound taken alone. On the other hand, evaluation of the influence of the antibiotic and adjuvant concentrations on the aerodynamic properties measured previously was made by fixing the duration of nebulization and varying the concentrations of the two compounds in the solution for nebulization. The aerodynamic characterization of the different aerosols generated at different concentrations of the two compounds showed median mass aerodynamic diameters ranging from 3.1 to 4.5 µm and FPFs varying from 52 to 59%, led to the conclusion that the concentration in active pharmaceutical ingredients does not influence the aerodynamic properties of the combination aerosol, since a FPFs greater than 50% is always encountered. This makes it possible to adapt the posology without influencing the aerodynamic properties and the particles capacity to reach the site of the infection in quantity needed for efficiency. The effect of the duration of nebulization on the aerodynamic parameters previously obtained for the combination aerosol generated by the PARI LC Plus nebulizer was evaluated by fixing the concentration of the two active components (doxycycline and NV716). The obtained results showed no variation of the aerodynamic diameter with the duration of nebulization, with Median Mass Aerodynamic Diameters MMADs ranging from 3.43 to 4 µm in accordance with standards required for therapeutic aerosols (MMAD less than 5 µm). On the other hand, the percentage of fine particles FPF is not influenced by the duration of nebulization (1, 3, 6 or 10

min) and remaining always in the same zone ranging from 50 to 60%. Thus, the obtained results allow us to conclude that the aerodynamic parameters of the antibiotic/adjuvant combination aerosol generated by the PARI LC Plus do not depend on the duration of nebulization and are reproducible over time, which avoids the absence of deposition of droplets in insufficient quantity at the desired site of action even if the patient does not accurately respect the required duration of nebulization.

Moreover, to study the influence of the nebulizing device and improve the aerodynamic properties of the doxycycline/NV716 combination aerosol two other nebulizers were chosen, in addition to the PARI LC Plus used previously, the PARI Sprint: a pneumatic air jet nebulizer belonging to the same nebulizers family as the PARI LC Plus, and the PARI eFlow: a vibrating mesh nebulizer. Thus, after fixing the concentration of active principles and the duration of nebulization we have characterized the different aerosols generated by the different nebulizers. It clearly appears that each type of nebulizer possesses its own specificity:

- The PARI LC Plus generated droplets with the smallest MMAD of 3.5 μm .
- The PARI Sprint gave the best results in terms of nebulization flow rate of 0.59 mL/min which allowed the nebulization in 6 min of 3.5 mL of the inhalation solution compared to 1.88 mL and 2.9 mL nebulised by the PARI LC Plus and the PARI eFlow respectively.
- The PARI eFlow with the PARI LC plus gave the highest percentage of fine particles of 60 % compared to 53% of FPF obtained by PARI Sprint.

The geometric standard deviation (GSD) of the particles aerodynamic diameter with a value of 1.8 for the PARI eFlow is lower than those obtained with the PARI LC Plus and the PARI Sprint (2.8 and 2.3, respectively) and results in a smaller distribution of the two compounds droplets (doxycycline and NV716) produced by the PARI eFlow at the different stages of the impactor. Otherwise, the mass distribution of the two compounds obtained with the PARI eFlow nebulizer shows a maximum of deposition on stage 4, average amounts deposited on stages 3 and 5, and small quantities on stages 2 and 6 while very small amounts were encountered on stages 1 and 7 of doxycycline and almost no trace of NV716 in stage 7.

The PARI LC Plus and PARI Sprint gave similar GSDs of 2.8 and 2.3 and allowed a wide dispersion of the two compounds on the different stages of the impactor, the maximum deposit encountered on the stage 4 but with a homogeneous distribution of the two compounds on the

other stages which is to obtain the synergy between doxycycline and the adjuvant NV716 for the efficiency of the combination. Additionally, we notice the presence of NV716 on all stages of the impactor even on stage 7 contrary to what is observed with the PARI eFlow.

Thus, the PARI Sprint nebulizer seems preferable for the delivery of our combination (Antibiotic-Adjuvant) since in addition to its ability to ensure a homogeneous distribution of the two compounds on the different stages of the NGI impactor required for the restoration of the Doxycycline activity by the NV716, it gives droplets with a MMAD suitable for pulmonary administration and allows the drug particles to reach the site of the infection. Furthermore, its high performance in terms of rapid nebulization (0.59 mL / min) will allow the patient to reduce the time of his nebulization session and consequently improve his quality of life.

Conclusion

Doxycycline/NV716 combination appears as a potent valuable drug for the treatment of *P. aeruginosa* pulmonary infections such as those encountered in cystic fibrosis patients. With new interpretations of the mechanism of action of non-cytotoxic compound NV716 and its active moiety doxycycline, new pharmaceutical development strategies could provide an adjunct pulmonary therapy to decrease the duration of treatment and improve the efficiency of the current drug regimen. Finally, polyaminoisoprenyl salt derivative NV716 was found suitable for aerosolization, and the morphology and droplets size were within the range suitable for pulmonary delivery. Our future work will deal with the optimization of the spray process, performance of studies to evaluate the *in vivo* efficacy, and tolerability of the doxycycline/NV716 combination in animals.

References

- Ariyananda, P., Agnew, J., Clarke, S., 1996. Aerosol delivery systems for bronchial asthma. *Postgrad. Med. J.* 72, 151-156.
- Aslam, B., Wang, W., Arshad, M.I., Khurshid, M., Muzammil, S., Rasool, M.H., Nisar, M.A., Alvi, R.F., Aslam, M.A., Qamar, M.U., 2018. Antibiotic resistance: a rundown of a global crisis. *Infection and drug resistance* 11, 1645.
- Borselli, D., Brunel, J.M., Gorgé, O., Bolla, J.M., 2019. Polyamino-isoprenyl derivatives as antibiotic adjuvants and motility inhibitors for *Bordetella bronchiseptica* porcine pulmonary infection treatment. *Front. Microbiol.* 10, 1771.
- Borselli, D., Lieutaud, A., Thefenne, H., Garnotel, E., Pagès, J.-M., Brunel, J.M., Bolla, J.-M., 2016. Polyamino-isoprenic derivatives block intrinsic resistance of *P. aeruginosa* to doxycycline and chloramphenicol *in vitro*. *PLoS One* 11.
- Bouza, E., Burillo, A., Munoz, P., 2002. Catheter-related infections: diagnosis and intravascular treatment. *Clin. Microbiol. Infect.* 8, 265-274.
- Carvalho, T.C., McConville, J.T., 2016. The function and performance of aqueous aerosol devices for inhalation therapy. *J. Pharm. Pharmacol.* 68, 556-578.

Carvalho, T.C., Peters, J.I., Williams III, R.O., 2011. Influence of particle size on regional lung deposition—what evidence is there? *Int. J. Pharm.* 406, 1-10.

Chan, J.G.Y., Wong, J., Zhou, Q.T., Leung, S.S.Y., Chan, H.-K., 2014. Advances in device and formulation technologies for pulmonary drug delivery. *AAPS PharmSciTech* 15, 882-897.

Cheng, Y.S., 2014. Mechanisms of pharmaceutical aerosol deposition in the respiratory tract. *AAPS PharmSciTech* 15, 630-640.

Dautzenberg, B., Becquemin, M., Chaumuzeau, J., Diot, P., Du GAT, P.L.M., 2007. Bonnes pratiques de l'aérosolthérapie par nébulisation. *Rev. Mal. Respir.* 24, 751-757.

Dautzenberg, B., Bonfils, P., Diot, P., Faurisson, F., Fauroux, B., 1998. Propositions de bonnes pratiques de l'aérosolthérapie par nébulisation. *Cahiers de kinésithérapie* 189, 29-36.

Dessanges, J., 1998. Nébulisation: principes et générateurs, *Ann. Fr. Anesth. Reanim.* Elsevier, pp. 275-305.

Douafer, H., Andrieu, V., Phanstiel IV, O., Brunel, J.M., 2019. Antibiotic adjuvants: make antibiotics great again! *J. Med. Chem.* 62, 8665-8681.

Ferron, G., 1994. Aerosol properties and lung deposition. *Eur. Respir. J.* 7, 1392-1394.

Groneberg, D., Witt, C., Wagner, U., Chung, K., Fischer, A., 2003. Fundamentals of pulmonary drug delivery. *Respir. Med.* 97, 382-387.

Le Brun, P., De Boer, A., Frijlink, H., Heijerman, H., 2000. A review of the technical aspects of drug nebulization. *Pharm. World Sci.* 22, 75-81.

Lemarié, E., Diot, P., 2005. Aérosolthérapie. *EMC-Médecine* 2, 56-65.

Livermore, D., 2004. The need for new antibiotics. *Clin. Microbiol. Infect.* 10, 1-9.

Manfredi, R., Nanetti, A., Ferri, M., Chiodo, F., 2000. *Pseudomonas* spp. complications in patients with HIV disease: an eight-year clinical and microbiological survey. *Eur. J. Epidemiol.* 16, 111-118.

McCallion, O., Taylor, K., Bridges, P., Thomas, M., Taylor, A., 1996. Jet nebulisers for pulmonary drug delivery. *Int. J. Pharm.* 130, 1-11.

Mercer, T., 1981. Production of therapeutic aerosols; principles and techniques. *Chest* 80, 813.

O'Callaghan, C., Barry, P.W., 1997. The science of nebulised drug delivery. *Thorax* 52, S31.

Pang, Z., Raudonis, R., Glick, B.R., Lin, T.-J., Cheng, Z., 2019. Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and alternative therapeutic strategies. *Biotechnol. Adv.* 37, 177-192.

Patton, J.S., Byron, P.R., 2007. Inhaling medicines: delivering drugs to the body through the lungs. *Nature reviews Drug discovery* 6, 67.

Payne, D.J., 2008. Desperately seeking new antibiotics. *Sci* 321, 1644-1645.

Peterson, E., Kaur, P., 2018. Antibiotic resistance mechanisms in bacteria: relationships between resistance determinants of antibiotic producers, environmental bacteria, and clinical pathogens. *Front. Microbiol.* 9, 2928.

Radan, C., 2017. L'aérosolthérapie dans le traitement des troubles respiratoires. *Actual. Pharm.* 56, 35-39.

Rangaraj, N., Pailla, S.R., Sampathi, S., 2019. Insight into pulmonary drug delivery: Mechanism of drug deposition to device characterization and regulatory requirements. *Pulmonary pharmacology & therapeutics* 54, 1.

Reychler, G., Dessanges, J., Vecellio, L., 2007. Aérosols: présent et futur. *Rev. Mal. Respir.* 24, 1013-1023.

Sharma, G., Rao, S., Bansal, A., Dang, S., Gupta, S., Gabrani, R., 2014. *Pseudomonas aeruginosa* biofilm: potential therapeutic targets. *Biologicals* 42, 1-7.

Thompson, P.J., 1998. Drug delivery to the small airways. *Am. J. Respir. Crit. Care Med.* 157, S199-S202.

Valderrey, A.D., Pozuelo, M.J., Jiménez, P.A., Maciá, M.D., Oliver, A., Rotger, R., 2010. Chronic colonization by *Pseudomonas aeruginosa* of patients with obstructive lung diseases: cystic fibrosis, bronchiectasis, and chronic obstructive pulmonary disease. *Diagn. Microbiol. Infect. Dis.* 68, 20-27.

Vivas, R., Barbosa, A.A.T., Dolabela, S.S., Jain, S., 2019. Multidrug-resistant bacteria and alternative methods to control them: An overview. *Microb. Drug Resist.* 25, 890-908.

Wikler, M.A., 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard. CLSI (NCCLS) 26, M7-A7.