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## Feasibility of an inhaled antibiotic/adjuvant dry powder combination using an experimental design approach

Hana Douafer<sup>1</sup>, Véronique Andrieu<sup>2</sup>, Emmanuel Wafo<sup>1</sup>, Michelle Sergent<sup>3</sup> and Jean Michel Brunel<sup>1\*</sup>

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

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

<sup>3</sup> Aix Marseille Univ, IMBE, UMR CNRS IRD Avignon Université, Site de l'Etoile, Marseille, France

### Abstract

The global increase of multidrug resistant bacteria and the lack of new classes of antibiotic especially those targeting Gram-negative pathogens are leaving the clinicians disarmed to treat numerous bacterial infections. Recently, the design of adjuvants able to enhance antibiotics activities appears to be one of the most promising investigated solutions to circumvent this problem. In this context, we have recently identified a new polyaminoisoprenyl derivative **NV716** able to potentiate, at a very low concentration the activity of doxycycline against resistant *P. aeruginosa* bacterial strains by increasing its intracellular concentration. In this study we will report an experimental protocol to optimize a dry powder for inhalation ensuring the simultaneous delivery of an antibiotic (doxycycline) and an adjuvant (the polyaminoisoprenyl derivative **NV716** since aerosol therapy could allow a rapid drug administration and target the respiratory system by avoiding the first pass effect and minimizing undesirable systemic effects. Thus, an experimental design was carried out permitting to identify the influence of several factors on the aerosolization efficiency of our combination and allowing us to find the right composition and manufacture leading to the best optimization of the simultaneous delivery of the two compounds in the form of an inhalable powder. More precisely, the powders of the two active ingredients were prepared by freeze drying and their aerosolization was improved by the addition of carrier particles of lactose inhalation grade. Under these conditions, the best formulation was defined by combining the optimal factors leading to the best aerodynamic properties' values (the lowest MMAD (Mass Median Aerodynamic Diameter) and the highest FPF (Fraction of Fine Particles)) without even using sophisticated engineering techniques. Finally, 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**

Pulmonary drug administration possesses several advantages, including optimal local delivery of the Active Pharmaceutical Ingredient (API) directly to its site of action in the lungs, rapid achievement of its pharmacological effect due to its rapid absorption provided by the large absorption surface area in the lungs and its rich vascularization.[1] In the case of antibiotics, reaching the optimal concentration at the infection site is essential for effective infection treatment.[2, 3] However, for some antibiotics (such as colistin), only low concentrations can be obtained at the infection site in the lungs after oral or parenteral administration.[4] Thus, inhalation administration of several antibiotics results in a significantly higher concentration in the respiratory tract than other routes of administration. Therefore, inhaled antibiotic therapies have increasingly been used for the treatment of respiratory infections.[5] This mode of local targeting minimizes undesirable side effects due to low systemic exposure also because doses can be reduced by up to 10 times compared to those required for an oral dose, has already proven in the management of lung infections in cystic fibrosis patients with tobramycin and colistin.[6] Recently we have highlighted the importance of this route of administration for the antibiotics delivery to treat respiratory infections.[7]

In this context, dry powder inhalers (DPIs) present many advantages over other inhalation devices such as the absence of propellant as well as their solid state conferring better chemical stability compared to liquid and metered dose inhaler aerosols.[8] Furthermore, they do not necessitate any external driving force for their operating and are easier to use for patients since they are portable and do not require coordination between inhalation and actuation.[9-11] Recently, emphasis has been placed on the development of dry powder inhalation [9] and a wide range of DPIs is currently available to maximize pulmonary drug delivery with low variability.[11] In general, dry powder formulations for inhalation are based on either free agglomerates of micronized drug particles with an aerodynamic diameter of less than 5  $\mu\text{m}$  or a mixture of such drug particles adhering to the surface of large carrier particles of an excipient, usually lactose,[11, 12] which purpose is to increase the fluidity and dispersion of API particles.[1] However, the major problem with antibiotics in both formulation and device design is their uniform delivery in high doses to the lungs. The difficulty arises from the high content of microscopically small, respirable APIs, whose increased cohesion between these drug particles lead to their decreased deagglomeration and promotes their adhesion to the wall of the inhaler, resulting in decreased flow properties and aerosol dispersion.[1] Thus, to overcome the cohesive nature of fine inhalable API particles, to improve their flow and to

promote their aerosolization, relatively coarse carrier particles are generally used.[13-15] There are different types of excipients that can be incorporated into DPIs, including lactose, mannitol, glucose, and strength control agents such as magnesium stearate.[16-18] The most commonly used excipient is lactose, often encountered in some marketed products, as fine particles of micro-metric size incorporated to increase the aerosol performance.[19-21] However, formulations based on carrier particles present certain limitations, such as bulkiness due to the incorporation of large amounts of carrier particles.[1] The increase in powder mass requires more energy (inspiratory air) to set the powder in motion and provide the forces necessary to its effective detachment and aerosolization.[1] In addition, such a high powder load can make the multi-dose inhaler voluminous and requires a greater number of inhalations to deliver the effective dose.[22] Therefore, minimizing the number of excipients used is an important factor in promoting patient compliance[13] as well as the adhesion of the drug particles to the carrier particles that should not be too strong to facilitate its detachment and ensure proper delivery to the lungs.[23]

In this context, it has been reported that addition of fine lactose in the dry powder increases the API fraction of fine particles (FPF)[24] by promoting breakage of API agglomerates, distributing them over the surface of the carrier, dampening the frictional and pressure forces as well as reducing the API-support adhesion forces.[1] Specifically, the binding of fine lactose particles to the active sites of the carrier could prevent fine drug particles from binding to these high-energy sites, then in counterpart bind to the comparatively smoother, low-energy surface regions of the carrier,[25] requiring relatively low forces for the API- carrier detachment.[1] We have recently reported the development and the aerodynamic characterization of a combination of an antibiotic (doxycycline) with an adjuvant (polyaminoisoprenyl derivative **NV716**) in the form of a solution for nebulization using a pneumatic nebulizer, intended for the treatment of respiratory infections caused by *Pseudomonas aeruginosa*. [26] The compound **NV716** acts as an adjuvant to doxycycline allowing the restoration of its antibacterial activity against naturally resistant *Pseudomonas aeruginosa* strains, resistance due to the outer-membrane impermeability. In this context, the mode of action of **NV716** has been demonstrated to be related to a dose-dependent perturbation of the outer bacterial membrane integrity inducing its permeabilization which facilitates the entry of intracellularly targeted antibiotics and thus the potentiation of their activities.[27]

The originality of this work comes from the simultaneous administration of two active ingredients acting in synergy, which means that the presence of both compounds in sufficient

quantity at the same time on the infection site is essential to achieve the expected pharmacological effect.

In the continuation of our studies, a new pharmaceutical form for inhalation based on this doxycycline/NV716 combination will be investigated to harness the inhalation dry powder benefits mentioned previously. Thus, the main objective is to evaluate the adaptability of this mixture to pulmonary administration in powder form as well as to identify the influence of several non-drug parameters on the aerosolization efficiency of this mixture, to optimize its delivery to its target action site (pulmonary infection site) in sufficient and suitable quantities ensuring the synergy between the antibiotic and the adjuvant.

## Materials

### 1. Inhalation device

The inhalation device used for the aerodynamic characterization in this study is the Dry Powder Inhaler DPI the HandiHaler® (HH, Boehringer Ingelheim, Germany), a single-dose breath activated inhaler, which means that the inhalation triggers the medication release. A capsule of powdered medication is placed in the device. Its operation is based on the powder release from the pierced capsule, following patient inspiration. The portable size, efficiency and convenience make the HandiHaler® a desirable method for inhalation treatment.

### 2. Active Pharmaceutical Ingredient (API)

The antibiotic used in this study is doxycycline and was purchased from Sigma -Aldrich (Saint-Quentin Fallavier, France). The adjuvant **NV716** is a polyaminoisoprenyl derivative synthesized according to a protocol elaborated in our laboratory (See **Supplementary Data SD1**) .[26, 28, 29]

### 3. Excipients

Three lactose grades (**Table 1**) were used in this study, they were kindly provided by DFE Pharma company (DFE Pharma, Germany).

**Table 1.** Characteristics of the different lactoses used in this study

Lactose type	Grade	Abbreviation	Description	D10 (µm)	D50 (µm)	D90 (µm)
<b>Lactohale® 206</b>	Large	LH206	Milled lactose with tightly controlled particle size	20 - 50	50 - 75	115 - 170
<b>Respitose® ML003</b>	Large	RESPI0003	Fine milled lactose	1 - 6	20 - 50	65 - 140
<b>Lactohale® 210</b>	Fine	LH210	Fine Lactose, milled with irregular shaped particles	2 - 4	14 - 19	35 - 50

D10 = diameter of 10% of the particles, measured by laser analysis. D50 = diameter of 50% of the particles. D90 = diameter of 90% of the particles.

## **Methods**

### **1. Powder preparation**

#### **a. Preparation of the solutions intended for lyophilization**

Three solutions have been prepared: the 1<sup>st</sup> contains doxycycline alone, the 2<sup>nd</sup> contains **NV716** alone and the 3<sup>rd</sup> contains a combination of doxycycline and **NV716** together. 20 mL of highly purified water (Millipore Synergy® UV, Laboratory Water Purification System) were used for each solution at a concentration of 3 mg/mL for doxycycline, 3 mg/mL for **NV716** and for the doxycycline: **NV716** (1.5 mg/mL: 1.5 mg/mL), respectively. All these solutions were stored in glass vials (300 mL) and then frozen at -80°C for 24 h.

#### **b. Freeze drying: Lyophilization**

Lyophilization was performed in a laboratory scale freeze dryer, which was first programmed at a temperature of -20 °C and a pressure of 5.17 mbar for 30 min (pre-heating).

Vials containing the solutions prepared as explained above and stored in the freezer at -80°C (for 12 hours) were quickly placed on the shelves of a Christ Alpha 1–4 LSCbasic Freeze Dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Germany). The primary drying was performed at a shelf temperature of -32°C and a vacuum pressure of 1 mbar for 20 hours, whereas for the secondary drying, the shelf temperature was fixed at -37 °C and it was performed for 40 hours.

After freeze-drying, the vials containing the freeze-dried powders were immediately hermetically sealed and stored in a desiccator under vacuum containing desiccant silica gel until their use for aerodynamic size characterization.

### **2. Capsule filling**

The different mixtures were filled individually into HPMC capsules (hydroxypropylmethylcellulose) size 3 (0.3 mL) using a manual capsule filler (**Supplementary Data SD2**). The target weight of 100% fill was set at 200 mg.

**Erreur ! Source du renvoi introuvable.** summarizes the rate of the different components of each mixture, their mixing order and duration according to the design of experiments. The filled capsules were immediately used for aerodynamic characterization, thus, no capsule was stored for more than 2 h, to avoid adsorption of moisture which could influence the powder behavior in inhalation and so the results reproducibility. Each capsule was weighed before and after the filling to evaluate uniformity of the filled mass.

### **3. Assessment of dry powder aerosol properties using the next generation impactor NGI**

The performance of freeze-dried powder aerosols was evaluated according to the European Pharmacopoeia monograph (Ph. Eur. 2.9.18) using the Next Generation Impactor (NGI, Copley, Nottingham, UK), which is a high-performance, precision and particle classifying impactor, composed of seven-stages plus a micro-orifice collector (MOC). It was coupled with a USP throat attached at its inlet to a mouthpiece adapter allowing the attachment of the used dry powder inhaler DPI: the "Handihaler®". The NGI was also equipped by a pre-separator according to the Pharmacopoeia monograph for dry powder inhalation aerodynamic characterization. Before each dispersion, a thin layer of petroleum jelly oil was applied to the different stages of the impactor (E1-E8) and to the pre-separator plate to minimize particle bounce.

For each dispersion, the DPI was loaded by a size 3 HPMC capsule filled with the powder to be inhaled. For all measurements, a flow rate of  $43 \pm 0.5 \text{ L min}^{-1}$  was applied through the impactor for 6 seconds, to pass a total of 4 L of air through the inhaler with a pressure drop of about 4 kPa applied through the inhaler.[30, 31] This flow rate was obtained using a flow regulator (Copley Scientific, UK) and a pump (Copley Scientific, UK) assembled at the NGI (Figure) and was measured by a Copley flow meter.

The capsule is punctured, the DPI is attached to the USP throat via the mouthpiece adapter, and then the pump is turned on to operate the powder aerosolization. A batch of several capsules (depending on the dose of API incorporated in the capsule) is dispersed at each pass to ensure that a quantifiable dose is obtained on each NGI stage.

After dispersion, the powder deposited on each stage as well as on the throat, the pre-separator and the DPI mouthpiece is recovered with a sufficient volume of ultrapure water. Different volumes of ultrapure water were used to recover the powder, in order to allow a quantifiable concentration of the two compounds (doxycycline and **NV716**): 5 mL for the throat, 15 mL for the pre-separator, 2 mL for the inhaler and 1 mL for each of the different NGI stages (Stage 1- Stage 8). The determination of the quantity deposited on each stage is done by mass coupled gas chromatographic determination. For each test, the results given correspond to the average of 2 measurements.

### **4. Gas chromatography–mass spectrometry GC–MS conditions**

Chromatographic analyses were performed using an Agilent Technologies 6890N Network GC System (Agilent 7683B Injector and autosampler) coupled to an Agilent 5973 Network mass spectrometer detector. The separation was achieved by using a 30 m × 0.25 mm internal diameter (I.D.) × 0.25µm film Thickness, Zebron ZB50 capillary column (Phenomenex). The injection volume was 1 µL, and the helium carrier gas flow was 1mL/min. 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, (hold time: 5 min). Selected ions for detection for each compound: **Doxycycline**: 393; 315; 255; 175;147; **NV716**: 205; 177; 159, 137.

### 5. Determination of the best coarse lactose

Inhalation powders consist of micronized particles less than 5 µm in diameter to ensure their arrival at their target site at deep lung levels. However, this small size leads to strong interparticle cohesion and poor flow of the powder which is unfavorable for administration by inhalation. Thus, it is necessary to add larger carrier particles to improve aerosolization and reduce cohesion between fine drug particles. In this case, the active ingredient particles smaller than 5 µm in diameter will detach from their carrier (lactose) and continue their way to the lungs through the airways to exert their therapeutic effect, while large lactose particles will be blocked in the ORL sphere and subsequently swallowed and eliminated through the digestive tract.

Initially, we have received two types of large lactose: LH206 lactose and RESPI003 lactose. Thus, we started with a pre-study to determine which size between the two improves the dispersibility of our compounds inhaled powders. For this, an experimental design was set up containing 2 factors: the Active Pharmaceutical Ingredient (API) with 3 levels (doxycycline, **NV716** and doxycycline+**NV716** together), and the type of lactose (Lactose) with 2 levels (lactose LH206 and lactose RESPI003) (**Table 2- a**).

**Table 2.** Summary of factors and levels of the pre-study designed for choosing the optimal size of large lactose (**a**), as well as the different mixtures tested (**b**)

<b>a</b>			<b>b</b>					
Levels number	Name / Value		Excipient		API			
Factor 1	3	API	Name	Particles size (µm)	(%)	Doxycycline (%)	NV716 (%)	
	Level 1	Doxycycline	LH206	75-95	95	5	0	M <sub>1</sub>
	Level 2	<b>NV716</b>				0	5	M <sub>2</sub>
	Level 3	Doxycycline+ <b>NV716</b>				5	5	M <sub>3</sub>

<b>Factor 2</b>	<b>2</b>	<b>Lactose</b>	RESPI003	20-50	95	5	0	M <sub>4</sub>
Level 1	LH206	0				5	M <sub>5</sub>	
Level 2	RESPI003	90			5	5	M <sub>6</sub>	

Thus, both types of lactose were used in association with doxycycline or **NV716** alone as well as with the doxycycline+**NV716** combination for the powder aerodynamic characterization using the NGI impactor (**Table 2- b**). Each mixture (**M**) was filled into a HPMC capsule of size 3 as explained above. The filled capsules were then tested in inhalation using the HandiHaler® DPI for the aerodynamic assessment.

## 6. Design of experiments

A design of experiments was carried out studying four parameters as proof of concept: the content of active ingredients (doxycycline + **NV716**), the capsule filling rate, the APIs rate, the lactose grade and the introduction order of the different compounds in the capsule.

The different factors were studied using two levels, except for the last one (the introduction order of the mixture components) which had four levels; the values used for each level and each factor and the design of experiments carried out are summarized in **Table 3**. The chosen experimental design is a screening design whose objective is to compare the behavior of the different levels for each factor.[32]

**Table 3.** Experimental domain summarizing the different factors studied and their levels (**a**) and experimental design (**b**) proposed to investigate the influence of several factors on the dry powder combination aerosolization effectiveness.

**a**

Factor	Levels Number	Name / Value	Abbreviation
<b>Factor 1</b>	<b>2</b>	<b>Filling rate</b>	<b>Filling</b>
	Level 1	50%	
	Level 2	100%	
<b>Factor 2</b>	<b>2</b>	<b>[Active Pharmaceutical Ingredient]</b>	<b>[API]</b>
	Level 1	2.5 %	
	Level 2	10%	
<b>Factor 3</b>	<b>2</b>	<b>Lactose</b>	<b>Lactose</b>
	Level 1	large	
	Level 2	Fine +large	
<b>Factor 4</b>	<b>6</b>	<b>Introduction order</b>	<b>Order</b>
	Level 1	Doxy 2mn+ <b>NV716</b> 2mn	
	Level 2	Doxy 4mn+ <b>NV716</b> 4mn	

**b**

Experience	Filling rate	[PA]	Lactose	Introduction order
<b>E1</b>	50%	2.5 %	large	Doxy 2mn+ <b>NV716</b> 2mn
<b>E2</b>	100%	2.5 %	fine +large	<b>NV716</b> 2mn+Doxy 2mn
<b>E3</b>	100%	10%	fine +large	Doxy 2mn+ <b>NV716</b> 2mn
<b>E4</b>	100%	2.5 %	large	Doxy 4mn+ <b>NV716</b> 4mn
<b>E5</b>	50%	10%	large	<b>NV716</b> 2mn+Doxy 2mn
<b>E6</b>	50%	2.5 %	large	<b>NV716</b> 4mn+Doxy 4mn
<b>E7</b>	50%	10%	Fine +large	Doxy 4mn+ <b>NV716</b> 4mn
<b>E8</b>	100%	10%	Fine +large	<b>NV716</b> 4mn+Doxy 4mn
<b>E9</b>	100%	10%	large	<b>NV716/L</b> 4mn Doxy/L 4mn
<b>E10</b>	100%	2.5 %	large	<b>NV716/L</b> 2mn Doxy/L 2mn
<b>E11</b>	50%	10%	Fine +large	<b>NV716/L</b> 2mn Doxy/L 2mn
<b>E12</b>	50%	2.5 %	Fine +large	<b>NV716/L</b> 4mn Doxy/L 4mn

Level 3	NV716 2mn+Doxy 2mn
Level 4	NV716 4mn+Doxy 4mn
Level 5	NV716/L 2mn Doxy/L 2mn
Level 6	NV716/L 4mn Doxy/L 4mn

## 7. Data analysis

The CITDAS® software (version 3.10 Wibu which meets the requirements of USP and Ph.Eur., Copley Scientific Ltd, Nottingham, UK) was used for the calculation of the different aerosolization performance parameters. **GraphPad Prism 8** software (GraphPad Software, Etats Unis) was used for creating the different graphs. Azurad software (AZURAD SAS, France) was used for the construction and the treatment of the experimental design.

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 between 15 and 100 L/min the CITDAS software calculates the Effective Cut-off Diameter for each stage using the following equation:

$$ECD' = ECD * (60 / Q) ^ x$$

where:

<i>NGI stages</i>	<i>x</i>
<i>Stage 1</i>	0,54
<i>Stage 2</i>	0,52
<i>Stage 3</i>	0,50
<i>Stage 4</i>	0,47
<i>Stage 5</i>	0,53
<i>Stage 6</i>	0,60
<i>Stage 7</i>	0,67

**ECD'** = the stage ECD at sampling flow rate Q

**ECD** = the stage ECD at a sampling flow rate of 60 litres/min

**Q** = the sampling flow rate [litres/min]

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

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

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

The Mass Median Aerodynamic Diameter (MMAD) is 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). MMAD is obtained by interpolating the aerodynamic diameter corresponding to 50% of particles undersize (or Probit 5).

## Results and discussion

The main objective of this study was the investigation on the influence of several factors on the aerosolization efficiency of an inhalation powder consisting of a mixture of two active ingredients (doxycycline and the **NV716** adjuvant) acting synergistically. The efficacy of compound **NV716** in restoring the antibacterial activity of doxycycline as well as the efficacy of the **NV716**/doxycycline combination against *Pseudomonas aeruginosa* strains has been previously studied.[26, 27, 33] Thus, in a first step we started by studying the suitability of the two compounds for inhalation as powder either separately or in combination, using two different types of lactose inhalation grade lactose as excipients. These two types of lactose differ by the size of their particles but are both considered to be wide range.

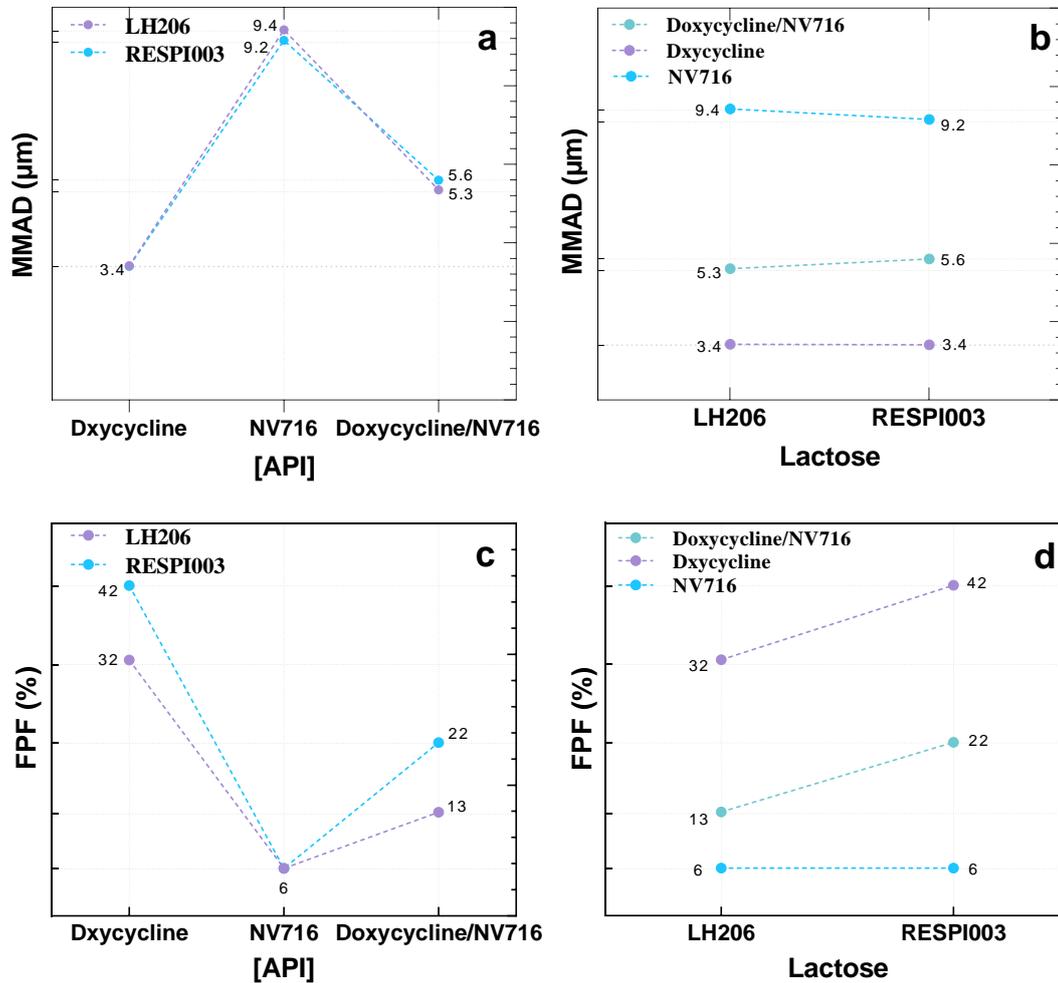
The results of the aerodynamic characterization (MMADs and FPFs) of the three powders (doxycycline alone, **NV716** alone and doxycycline + **NV716**) delivered in combination with large lactose LH206 or lactose RESPI003 are summarized in **Table 4**.

**Table 4.** Aerodynamic parameters of aerosols involving doxycycline, **NV716** alone or in combination, in association with lactose LH206 or RESPI003

	Mass Median Aerodynamic Diameter ( $\mu\text{m}$ )		Fine Particle Fraction (%)	
	LH206	RESPI003	LH206	RESPI003
Doxycycline alone	3.4	3.4	33	42
<b>NV716</b> alone	9.4	9.2	6	6
Doxycycline + <b>NV716</b>	5.3	5.6	13	22

Thus, the use of lactose LH206 resulted in MMADs of 3.4, 9.4 and 5.3  $\mu\text{m}$  and FPFs of 33, 6 and 13 % for the doxycycline powder alone, the adjuvant **NV716** alone and for the doxycycline/**NV716** combination, respectively (**Table 4**). On the other hand, addition of lactose RESPI003 to the powder of doxycycline or **NV716** alone as well as the doxycycline/**NV716** combination provided FPFs of 42, 6 and 22 % and MMADs of 3.4, 9.2 and 5.6  $\mu\text{m}$ , respectively. More precisely, **Figure 1** highlights the most important results obtained for the aerodynamic characterization of the two compounds powders particles

(doxycycline and **NV716**) delivered separately or in combination with the two types of large lactose LH206 or RESPI003.



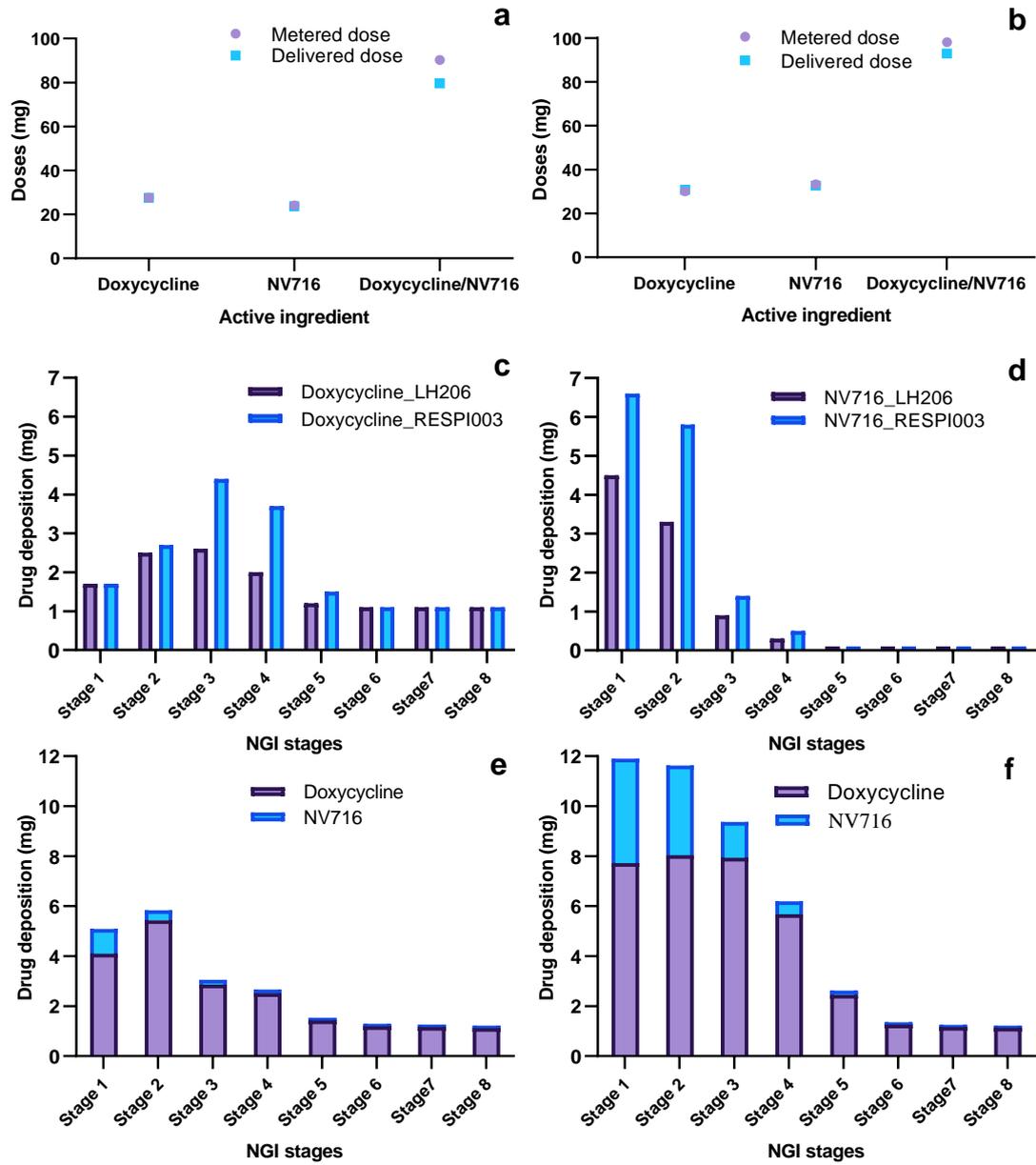
**Figure 1.** First-order interaction effects of MMADs according to the type of large lactose (a) or the used API (b). First-order interaction effects of FPFs according to the large lactose (c) or the used API (d).

As shown in **Figure 1-a**, the doxycycline powder gave MMAD of 3.4  $\mu\text{m}$  regardless of the type of lactose used whereas the **NV716** powder presented a MMAD of 9.4 and 9.2  $\mu\text{m}$  by using lactose LH206 or RESPI003, respectively. At the same time, the doxycycline/**NV716** combination lead in the presence of lactose LH206 or RESPI003 to MMAD values ranging from 5.3 and 5.6  $\mu\text{m}$ , respectively. Thus, it clearly appears that a change in large lactose used (two different particle sizes) did not influence the aerodynamic diameter of the three powders tested.

In addition, as shown in **Figure 1-b**, for this response (MMAD: the first response studied by the experimental design), the only influencing factor is the API. The type of large lactose does not influence the MMAD particle diameter: the results obtained with the two types of lactose are similar. However, it can be observed that the lowest response is obtained with doxycycline alone and the highest one is with the adjuvant **NV716** alone whereas the combination of the two powders lead to an intermediate result (**Figure 1-b**).

The Fine Particle Fraction obtained with the different mixtures of the two compounds with the two types of coarse lactose are presented in **Figure 1-c/d**Erreur ! Source du renvoi introuvable.. The formulations based on lactose LH206 gave FPFs of 33, 6 and 13 % for the powder of doxycycline alone, **NV716** alone and the doxycycline/**NV716** combination, respectively. At the same time, the use of RESPI003 lactose resulted in FPFs of 42 % for doxycycline alone, 6 % for **NV716** and 22 % for the doxycycline/**NV716** mixture. From these results it can be noted that even if RESPI003 does not influence the FPF obtained for **NV716**, its use allowed a clear improvement in the percentage of fine particles obtained with doxycycline alone and with the doxycycline/**NV716** combination.

In addition, **Figure 1-c** confirms that in the case of the **NV176** adjuvant powder, the lactose grade has no influence on the FPF (%). Nevertheless, it is noteworthy that the type of lactose used in the case of doxycycline powder alone or of the antibiotic/adjuvant combination has a significant effect on the percentage of fine particles as encountered for lactose RESPI003 allowing an increase of the FPF (**Figure 1-d**),.



**Figure 2.** Metered and delivered doses obtained from the different mixtures of the two compounds with lactose LH206 (a) or RESPI003 (b). Next Generation Impactor (NGI) deposition patterns of the two compounds delivered separately (c-d) or together (e-f) in mixture with lactose LH206 (e) or RESPI003 (f)

On the other hand, as shown in **Figure 2-a/b**, lactose RESPI003 allows the delivery of higher doses compared to lactose LH206, and this for the powders of the two compounds separately or in combination. Furthermore, the lactose RESPI003 (**Figure 2-b**) allows for less loss (difference between metered and delivered dose) compared to the lactose LH206 especially in the case of doxycycline/**NV716** combination.

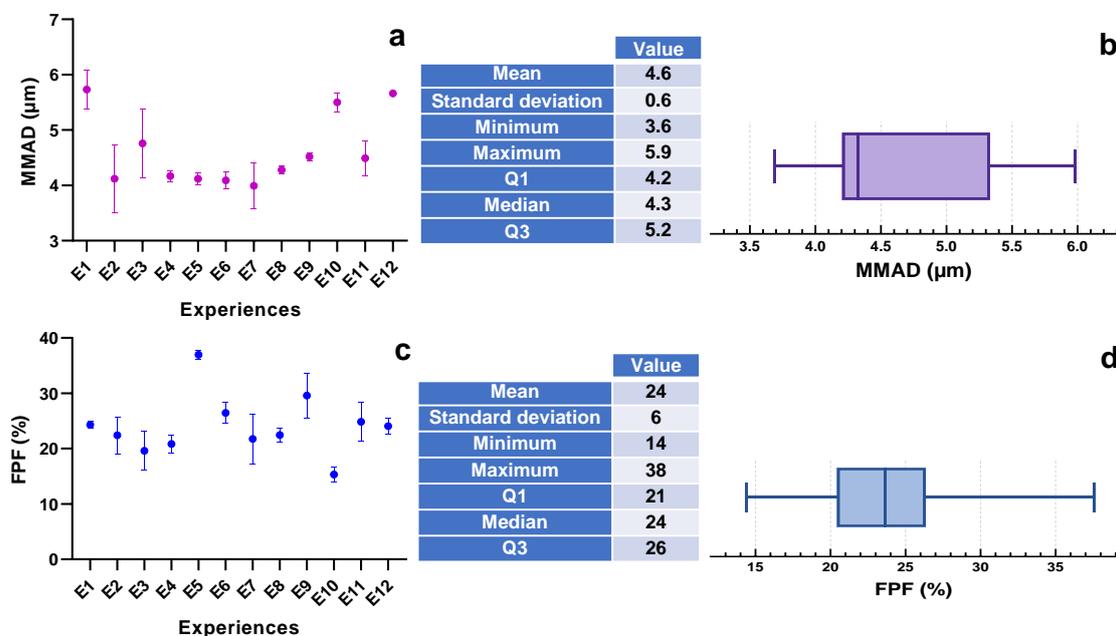
The powder deposition profiles on the different NGI stages of the two compounds delivered separately or as a combination using lactose LH206 or RESPI003 as excipient are grouped in **Figure 2 (c-d-e-f)**. The two types of large lactose gave a Gaussian particles distribution of the different powders tested on the impactor stages with maximum of deposition on: stage 2 for the adjuvant **NV716** powder and its mixture with doxycycline and stage 3 for doxycycline powder. However, for all the mixtures tested lactose RESPI003 allows a particles deposit higher than that obtained with LH206. Furthermore, for the formulation based on the mixture of doxycycline with **NV716**, RESPI003 allows a high deposition of both compounds on the NGI stages compared to those obtained with LH206. Moreover, in the case of **NV716** powder, we notice that RESPI003 facilitates its dispersion on the different stages leading to a higher deposited mass compared to those obtained using LH206, thus improving the ratio between the two compounds (doxycycline and **NV716**) on each stage, which is highly recommended in our case to ensure the synergy of the antibacterial activity between the two compounds. Based on all these results, the mixture obtained with RESPI003 lactose appears less cohesive compared to that obtained with L206 allowing an easier detachment of the fine particles.

According to the results obtained in this preliminary study, we can conclude that the lactose RESPI003 improves the inhalation powders fluidity which facilitates particles deagglomeration and it is thus suitable for use in the rest of our dry powder aerosolization optimization study to achieve an optimal aerodynamic particles behavior.

In a second part, we have focused our attention on the optimization of the dry powder combination of the two compounds (doxycycline and **NV716**) delivered together.

We aimed to improve the combination inhalation behavior by following an experimental approach using two lactose grades (large lactose alone and mixture of large lactose with fine lactose) and by modifying factors other than the excipients involved in the formulation. This was mainly done to limit the use of reagents and excipients that can harm the respiratory tract already in pathological state. Therefore, the influence of the capsule filling rate, the APIs rate, the lactose grade and the introduction order of the different components of the mixture were studied in relation to the Mass Median Aerodynamic Diameter MMAD and the Fine Particle Fraction FPF. This approach makes it possible to screen for factors affecting the aerodynamic

behavior and dispersibility of the two compounds combination and to understand how they may influence the efficacy and the deposit site of the inhalation powder in the respiratory tract. The results of the aerodynamic characterization of the different powder mixtures tested according to the previously detailed experimental design are summarized in **Figure 3**.



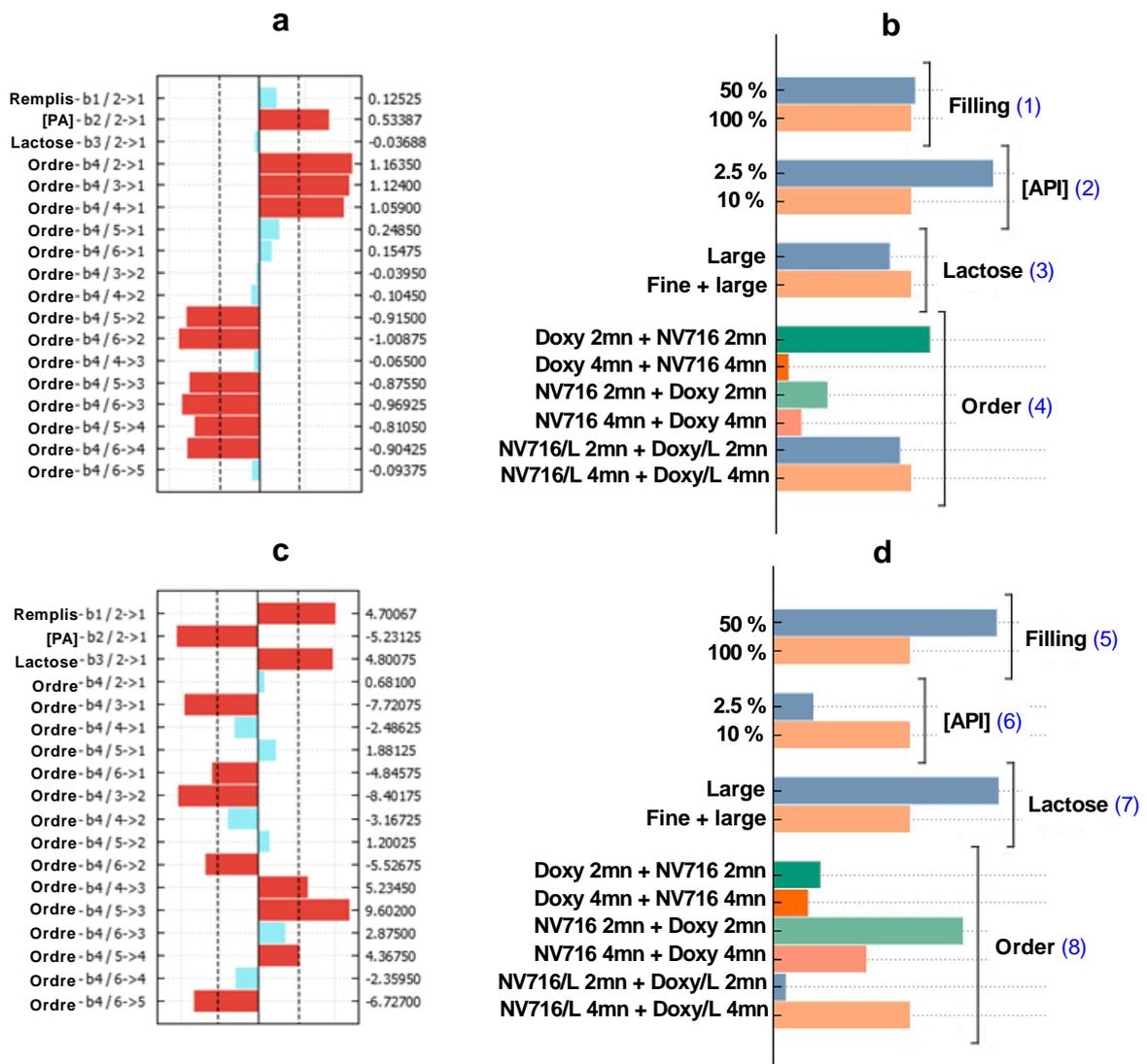
**Figure 3.** Aerodynamic characterization parameters MMADs (a) and FPFs (c) of the aerosols involving the combination of the lyophilized powders of doxycycline with that of NV716 according to different factors. Box Plots for MMADs (b) and FPFs (d) responses graphically represent their range of variation.

The variations of the different factors studied by the experimental design resulted in a wide range of MMADs between 3.6 and 5.9  $\mu\text{m}$  with an MMAD average of 4.3  $\mu\text{m}$ . In addition, the average FPF obtained was 24 %, the lowest FPF obtained was 14 % while the highest percentage of fine particles obtained was 38 %. Thus, according to these results we can notice a difference in the obtained MMADs, some are suitable for pulmonary administration (inferior to 5  $\mu\text{m}$ ), while others are above the recommended standards for inhaled treatment (superior to 5  $\mu\text{m}$ ). In addition, there is a huge difference between the percentage of fine particles ranging from 14 to 38%, which could dramatically influence the concentration of active ingredients at their target sites in the deep areas of the lungs, and consequently their antibacterial activity and therapeutic efficiency.

The variation in the dry powders aerodynamic properties obtained reflects the influence of the different factors studied, and underlines the possibility of optimizing the aerosolization of

powders designed for inhalation without even using sophisticated inhaled particle engineering techniques [34] such as the preparation of Large Porous Particles for Pulmonary Delivery [35, 36]) as well as the importance to take them into consideration when developing DPI.

From the experimental results of the 12 experiments given by the design of experiments, it's possible to evaluate the "weight" of each factor to quantify their influence on the aerodynamic properties. To make easier the analysis of the results, graphs were represented to compare the behavior of the different values tested for each factor. The interpretation of these graphs required statistical tools to test how significant is the influence of the factors. These statistical tests used the experimental variance calculated from the replicates. In **Figure 4a** and **4c**, were presented graphs with the variation of MMAD and FPF caused by the change in levels (2 or 6 levels) for each factor. **Figure 4b** and **4d** show a synthesis of the behavior of the factors on the aerodynamic properties of the powder mixtures.



**Figure 4.** Analysis of the impact of several factors on the aerodynamic characteristics: MMAD (a, b) and FPF (c, d) of the combination of doxycycline with NV716 tested in inhalation. experiments.

### **1. Effect of the capsule fill rate on the aerosolization efficiency of doxycycline/NV716 combination powder**

To evaluate the impact of the capsule fill rate on the ease of dispersion and aerosolization of the combination inhalation powder, two fill rates were chosen: 50% and 100% of the capsule volume. **Figure 4-a-1** indicates that the fill rate does not influence the MMAD of the inhaled particles whereas the decrease of the fill rate to 50% is associated with a clear increase in FPFs (**Figure 4-d-5**). This may be due to an increase of the free space inside the capsule allowing a better deagglomeration of the formulation facilitating the powder dispersion and the detachment of the fine particles from their support (lactose). Thus, to increase the FPF rate, a half-filling of the capsules should be considered.

### **2. Influence of the active ingredients rate**

The influence of the APIs rate on the aerodynamic profile of the powder mixture (doxycycline/NV716) was studied by setting two APIs rates: 2.5% and 10% of the mixture of the two powders in regard to the total volume of the powder in the capsule (total volume  $V_t = V_{API} + V_{Lactose}$ ). As shown in **Figure 4-a-2**, the API level has an influence on the MMAD of the inhaled particles which is inversely proportional with this API level, since a 2.5% API level is associated with an increase in MMAD contrary to what was obtained with a 10% API level. On the other hand, the API rate is directly proportional to the fine particle fraction FPF since a high rate in API (10%) results in an increase in the FPF while its decrease to 2.5% allows the FPF decrease (**Figure 4-b-6**).

This result is particularly advantageous in our case, since an optimal result consists in a low MMAD (less than 5  $\mu\text{m}$ ) allowing an optimal deposition in the deep parts of the lungs (the site of bacterial infections) and a high FPF ensuring a sufficient concentration (above the Minimal Inhibitory Concentration (MIC) of the antibiotic) at the infection site. Thus, from these results we must notice that for an optimal delivery of our powder mixture, a high API level of 10% is strongly recommended, allowing a decrease in MMAD and an increase in FPF.

### **3. Influence of the carrier excipient**

The first part of this study allowed us to select the optimal size of the large lactose. In this second one, two excipients grades were used: the first is the lactose RESPI003 alone chosen

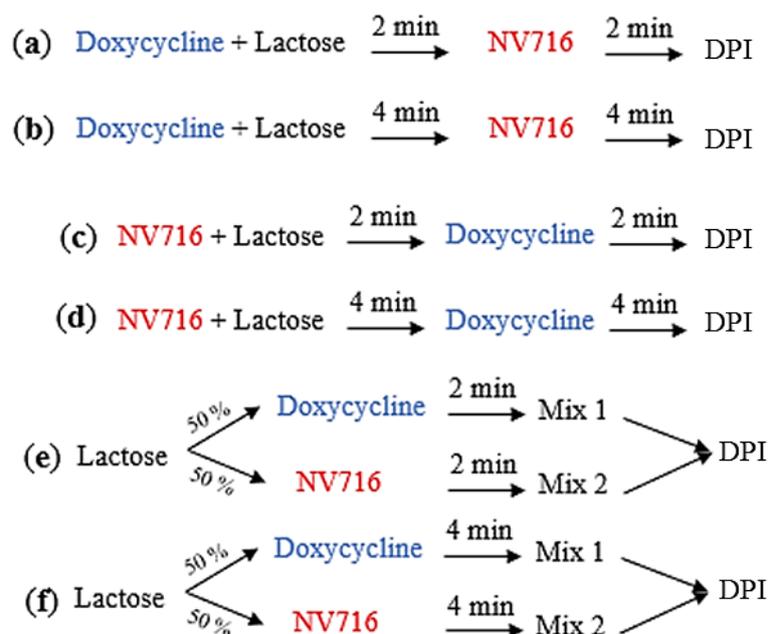
according to the results previously obtained whereas a mixture of lactose RESPI003 with another type of fine lactose LH210 as the second choice. Indeed, the addition of fine lactose particles is generally recommended to increase the detachment capacity of the active ingredient from its carrier, as these fine lactose particles will occupy the high-energy active sites on the surface of the large lactose particles. Thus, it is expected that the API will adsorb on the less powerful sites, ensuring a less strong fixation and thus a more effective detachment *in vivo*.

As illustrated in **Figure 4-a-3**, it clearly appears that the lactose grade does not impact the MMAD of the powder particles but has a direct impact on the FPF. More precisely, the two lactose grades tested lead to similar MMAD responses, while the lactose RESPI003 alone was more favorable for the optimization of the FPF level compared to its mixture with fine lactose.

#### 4. Influence of the introduction order of the different ingredients and duration of their mixing

The last factor studied is the order of introduction of the active principles and the duration of mixing as well as the introduction of the different powders into the final blend delivered using the dry powder inhaler.

Thus, six levels have been studied for this factor, they are summarized in **Figure 5**.



**Figure 5.** Order and duration of the two compounds mix with lactose to obtain the Dry Powder Inhalation (DPI). Mixing doxycycline with lactose for 2 min then adding NV716 (mix for 2 min) (a). The same previous order, extending each mix duration to 4 min (b). Mix of NV716 with lactose (2 min) then adding doxycycline

(for 2 min) (c). The same precedent order, mixing time of 4 min (d). Dividing the amount of lactose into two equal parts, each part is mixed with each of the two compounds for 2 min, mixing the two blends (e). The same previous order performed for 4 min for each step (f)

According to the results obtained, the introduction order of the different components in the final mixture constituting the powder for inhalation delivery has an impact on the MMAD and also on the FPF (Figure 4).

According to Figure 4-b-4, the order that gives the lowest MMAD is the one including the mixing of doxycycline first with lactose (for 4 min) then the addition of NV716 (Figure 5-b). In addition, the mix of NV716 first with lactose (for 4 min) then the addition of doxycycline (mixing for 4 min) also results in the decrease of MMAD (Figure 5-d) suggesting that the order in which the two compounds are mixed with the lactose has little influence, however the duration of 4 min is more favorable for the decrease of MMAD. On the other hand, the mix of the doxycycline first with the lactose for 2 min then adding the NV716 lead to the highest MMAD and is the most unfavorable order (Figure 5-a). Dividing the lactose into two parts and mixing them with each compound separately for 2 or 4 min showed similar effects ensuring a small increase of MMAD.

Concerning the FPF (Figure 4-d-8), the worst order was encountered with the mix of each compound separately with the lactose for 2 min and then mixing the two mixtures (Figure 5-e), since it gives the lowest FPF response.

In addition, the two orders starting with doxycycline whether mixed for 2 min or 4 min each time were also unfavorable by presenting low FPFs (Figure 5-a or Figure 5-b). On the other hand, the 3 orders starting with NV716 (during 2 or 4 min or its mixture with half the amount of lactose during 4 min) allow the increase of the FPF and are recommended for FPF optimization (Figure 5-c, Figure 5-d and Figure 5-f). Thus, for this latter parameter, the introduction order is more influential in relation to the mixing duration, and the introduction of NV7116 first constitutes the most favorable condition allowing the increase of the FPF.

Taking together the results obtained for both responses (MMAD and FPF) lead to the conclusion that the optimal order of introduction for our formulation includes the mixing of NV716 first with lactose for 2 min and then the addition of doxycycline for 2 min more since it results in a decrease of MMAD and an increase of FPF.

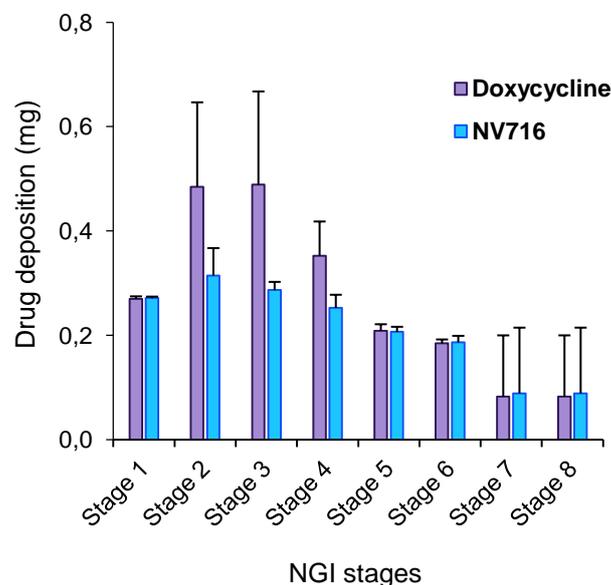
## 5. The optimal mixture

According to the results obtained by the different tests carried out previously giving the best aerodynamic properties we have established as outlined in **Table 5** the optimal conditions mixture for the two compounds ensuring their best aerodynamic profile in inhalation.

**Table 5.** Optimal formulation of the antibiotic/adjuvant combination and its aerodynamic properties

Capsule filling rate	2.5 %
Active Pharmaceutical Ingredient rate	10 %
Lactose	Large lactose (RESPI003)
Introduction order	<b>NV716 2mn + Doxy 2mn</b>
MMAD ( $\mu\text{m}$ )	<b>4.1</b>
FPF (%)	<b>38</b>

Thus, the combination of the optimized parameters identified above resulted in MMAD of 4.12  $\mu\text{m}$  which meets the recommended standard for inhaled treatments (5  $\mu\text{m}$ ) and allows the particles to reach deep regions of the lungs. In addition, this formula demonstrated a suitable FPF of 37% (the highest FPF obtained) allowing the two APIs to reach concentrations necessary for their antibacterial activities at the infection site.



**Figure 6.** Deposition patterns on the NGI stages of lyophilized doxycycline and **NV716** delivered simultaneously according to the optimal parameters

On the other hand, it clearly appears as illustrated in **Figure 6** that the two compounds are well distributed over the different NGI stages. Moreover, the deposited quantities are similar for most of the stages (Stages: 1,5, 6, 7 and 8) and very close for the others (stages 2, 3 and 4). This is particularly important for the combination to ensure the doxycycline activity potentialization by **NV716** to achieve the desired pharmacological effect.

## **Conclusion**

The objective of this study was the development of an experimental protocol to optimize the development of a dry powder for inhalation ensuring the simultaneous delivery of two active ingredients that act synergistically: an antibiotic (doxycycline) and an adjuvant (the polyaminoisoprenyl derivative **NV716**). Thus, an experimental design was carried out allowing the identification of the impact of several factors on the aerodynamic properties of the inhalation powder mixture, notably the MMAD, the FPF and the site of deposition of the active ingredients in the respiratory tract. The results obtained demonstrated the influence of these factors on the aerosolization efficiency of our APIs combination, some of which allow the improvement of their aerodynamic behavior such as the filling rate of 50% of the capsule volume, the API rate of 10 % and large lactose RESPI003 used alone. By contrast, others lead to an increase in MMAD or a decrease in FPF and are therefore unfavorable for our formulation (such as the 100% capsule filling and the APIs rate of 2.5%). Finally, the combination of the different favorable parameters allowed us to find the right composition and manufacture leading to the best optimization of the simultaneous delivery of the two compounds in the form of an inhalable powder without even using particle engineering sophisticated techniques or using a panel of excipients (as flow enhancers and lubricants) that can adversely affect the airways already in a pathological state.

Through this study, we have demonstrated that the dose of the drugs delivered *in vitro* as a powder provides the **NV716**/doxycycline ratio of 1:1 on most of the impactor stages, which is necessary to ensure the **NV716**/doxycycline combination effectiveness. However, extensive *in vivo* pharmacokinetic studies must be conducted to determine the therapeutic dose allowing the pharmacological effect. *In vivo* research are now under current investigations to evaluate the **NV716**/doxycycline combination efficiency and safety in a healthy rat animal model as well as including the determination of the optimal dose to be administered to provide the efficient concentration at the infection site while staying away from the zone of toxicity and adverse effects.

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