UNIVERSITÀ DEGLI STUDI DI SALERNO

DIFARMA

UNIVERSITÀ DEGLI STUDI DI SALERNO
Dipartimento di Farmacia

PhD Program
in **Drug Discovery and Development**
XXX Cycle — Academic Year 2017/2018

PhD Thesis in

Drug delivery to the lung in Cystic Fibrosis: deposition, dissolution, mucus interaction and microbiological evaluation of dry powder inhalers.

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Summary

LIST OF PUBLICATIONS

Papers

Stigliani M., **Manniello M.D.**, Zegarra-Moran O., Galietta L., Minicucci L., Casciaro R., Garofalo E., Incarnato L., Aquino R.P., Del Gaudio P., Russo P., 2016. Rheological Properties of Cystic Fibrosis Bronchial Secretion and *in Vitro* Drug Permeation Study: The Effect of Sodium Bicarbonate. J. Aerosol Med. Pulm. Drug Deliv. 29, jamp.2015.1228. doi:10.1089/jamp.2015.1228

Manniello M.D., Del Gaudio P., Porta A., Aquino R.P., Russo P., 2016. Aerodynamic properties, solubility and in vitro antibacterial efficacy of dry powders prepared by spray drying: Clarithromycin versus its hydrochloride salt. Eur. J. Pharm. Biopharm. 104. doi:10.1016/j.ejpb.2016.04.009

Manniello M.D., Del Gaudio P., Aquino R.P., Russo P., 2017. Clarithromycin and N-Acetylcysteine co-spray-dried powders for pulmonary drug delivery: A focus on drug solubility. Int. J. Pharm. doi:10.1016/j.ijpharm.2017.03.079

Tiozzo Fasiolo, L., **Manniello, M.D.**, Tratta, E., Buttini, F., Rossi, A., Sonvico, F., Bortolotti, F., Russo, P., Colombo, G., 2017. Opportunity and challenges of nasal powders: Drug formulation and delivery. Eur. J. Pharm. Sci. doi:10.1016/j.ejps.2017.09.027

List of communications

M.D. Manniello, A. Simonetti, P. Del Gaudio, R.P. Aquino, P. Russo "Drug solubility and aerodynamic properties of dry powders prepared by spray drying: clarithromycin versus its hydrochloride salt". 9th A.It.U.N. Annual Meeting from food to pharma: the polyhedral nature of polymers". University of Milan, 25th – 27th May 2015.

Russo P., **Manniello M.D**., Simonetti A., Petrone A.M., Porta A., Del Gaudio P., Aquino R.P.
"Aerodynamic Properties and Drug Solubility of Dry Powders Prepared by Spray Drying: Clarithromycin *Versus* its Hydrochloride Salt". 3rd International TB meeting Inhaled Therapies for Tuberculosis and other Infectious Diseases" – University of Parma, 14th – 16th October 2015.

M.D. Manniello, C. Pascale, R.P. Aquino, P. Del Gaudio, P. Russo. "Co-spray-dried Clarithromycin and N-Acetylcysteine Powders for Cystic Fibrosis Patients: Technological Properties and Aerodynamic Behaviour". Oral presentation at 11th Central European Symposium on Pharmaceutical Technology (CESPT2016); Belgrade (Serbia), 22nd – 24th September 2016.

F. Iadaresta, **M.D. Manniello**, C. Ostman, C. Crescenzi, J. Holmback, P. Russo. "Aromatic Amines in Textile as Potential Threat for Human Health" XXVI Congresso della Società Chimica Italiana. Paestum, 10th – 14th September 2017.

P. Russo, **M.D. Manniello**, L.F. Giordano, P. Del Gaudio, R.P. Aquino. "Clarithromycin dry powders for inhalation: A focus on drug solubility". A.It.Un. 11th Annual meeting. Padua, 11th and 12th May 2017.

L. Tiozzo Fasiolo, **M.D. Manniello**, L. F. Giordano, P. Russo and G. Colombo "Nano and mini spray-dried microparticle powder agglomerates for nose-to-brain delivery of flurbiprofen". A.It.Un. 11th Annual meeting. Padua, 11th and 12th May 2017.

THE PhD PROGRAM: AIMS AND OUTLINE

The inhalation therapy consists of a direct administration of drugs to the lung, particularly to take care of the pulmonary diseases. Indeed, a formulation for inhalation represents a valid option in the lung disease management of Cystic Fibrosis (CF), an inherited disorder that affects the normal function of the exocrine mucosal glands. Nonetheless, the stasis of the airways secretions leads to a worsening of the pulmonary functions, providing also the basis for the colonisation by resistant bacteria, in particular by *Staphylococcus aureus* and *Pseudomonas aeruginosa* species.

Using human bronchial sputum samples, obtained from CF patients of the *Gaslini Institute* of Genoa, the primary aim of my PhD program was to study the rheological behaviour of the CF sputum samples and the effect on it of a sodium bicarbonate solution.

Then, to understand the mechanisms that occur when inhaled micro- and nano-particles are deposited on the mucus and to check the ability of the drug to penetrate through CF mucus, the dissolution and permeation properties through CF sputum of ketoprofen lysinate from a previously developed Dry Powder Inhaler (DPI) was evaluated.

A common, but also a beneficial symptomatic treatment of CF, is represented by the administration of antibiotics, with the aim to improve the respiratory functions and slow down as much as possible the onset of the pulmonary worsening. Compared to liquid aerosol formulations, the Dry Powder for Inhalation (DPI) has some key advantages: a DPI is long-term stable because

the active pharmaceutical ingredient (API) is in solid form (dry powder) and pre-metered in a rigid capsule. A DPI is also solvent and propellant-free and may deliver the API directly to the lower airways and in high doses.

The main aim was also to design, produce and characterize an antibiotic DPI for the symptomatic treatment of the CF lung disease, taking into account the peculiarities of the pathology. For this purpose, the macrolide antibiotic clarithromycin (CLM) was selected among other APIs. It is well-known, in fact, that this semi-synthetic macrolide shows also immunomodulatory effects, together with the ability to attenuate the quorum sensing (cell-to-cell signalling), as well as to interfere with the virulence factors expression. These scientific evidences contributed for years to a proper credit towards this class of antibiotics.

A drug formulated as a dry powder for inhalation must accumulate in the site of action and, to exert its pharmacological effect, has to dissolve within the fluids of the lung, being this latter a very important issue for a CF patient. (Hasted J. et al., 2016). About this, pure CLM is characterised by a very poor water solubility, which can impair the local drug activity after the deposition. Therefore, the drug wettability and the dissolution process were enhanced by CLM salification which should confer a good distribution of the drug into the mucus without increasing the risk of absorption. It is reported, in fact, that ionized molecules interact with lipids and proteins surrounding the aqueous pores, lowering their absorption rate (Velaga S. et al., 2017). To ionize the dimethyl-amino group of the CLM molecule, small amounts of hydrochloric acid 1M were firstly used (Parvez M. et al, 2000). Secondly, the acidic moiety of N-Acetylcysteine, a well-known mucolytic drug, was selected to salify the CLM. The ionization, together with the fine-tuning of the spray drying process used to produce the fine particles,

contributed to achieve a good aerodynamic behaviour of CLAHCl and CLANAC powders, and to enhance the aqueous solubility and the dissolution process of CLM.

Finally, the CLM powders were tested to evaluate several aspects of the macrolide antibiotic towards the CF respiratory isolates, especially against *Pseudomonas aeruginosa* species, a common bacterial pathogen that colonise the lung of the CF patients that is able to trigger recalcitrant infections. Otherwise, the use of prophylactic anti-staphylococcal antibiotics has been shown to reduce clinical symptoms, and a need for additional antibiotics in the first two years of life was recognised (Smyth A.R. and Rosenfeld M., 2017). Among them, CLM is considered an acceptable alternative to treat *S. aureus* respiratory infections in children, compared to other old-generation macrolides (Scheld W. et al., 1992).

Through the minimal inhibitory concentration assay (MIC), the maintenance of the antibiotic's activity of CLM salt-form, when formulated as a DPI (CLAHCl and CLANAC formulations), was assessed using *Pseudomonas aeruginosa* and *Staphylococcus aureus* respiratory isolates. At the same time, the susceptibility of *P. aeruginosa* was examined in real-time by testing the antibiotic activity on the growth kinetics.

Moreover, the lowest dose of CLM able to inhibit the visible growth of *P. aeruginosa* from a *pre-formed* biofilm was investigated (MBEC assay).

INTRODUCTION

Chapter 1

PHYSIOPATHOLOGY OF CYSTIC FIBROSIS: CAUSES AND EFFECTS OF THE CFTR DYSFUNCTION IN THE LUNG

1.1 Cystic Fibrosis disease

1.1.1 Background of Cystic Fibrosis disease.

During the American Paediatric Society meeting in 1938, Cystic Fibrosis (CF) was described for the first time by Dorothy Andersen as an inherited autosomal recessive disorder and exploited as a *"fibrocystic disease of the pancreas"* (Andersen D., 1938; Cutting G.R., 2015).

About fifty years later, the defective gene responsible was identified. The CF disease is caused by mutations in a single gene on the long arm of chromosome 7, encoding the *cystic fibrosis transmembrane conductance regulator* (*CFTR)*, a protein constituted of five domains: two membrane-spanning domains, two nucleotide binding domains, and a unique regulatory domain (Riordan J.R. et al., 1989).

During the '90s, the basis for a better understanding of the structure and function of the CFTR transmembrane protein were set (Gibson R. et al., 2003) (Figure 1).

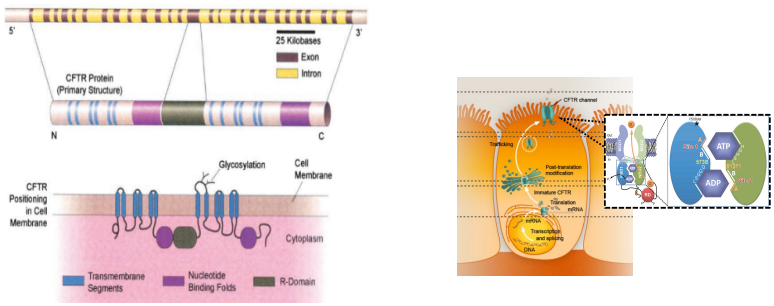

Figure 1. Schematic representation of the cftr gene and the CFTR mutant protein (F508del).

1.1.2 The CFTR dysfunction.

To date, about 1,900 different mutations in the CFTR gene have been reported. According to their effect on the synthesis, or on the function of the CFTR protein, the mutations are grouped into five classes (Figure 2, © 2016 Cystic Fibrosis foundation).

The most common mutation is part of the II class (indicated with ΔF508 or F508del), and consists in a deletion of three nucleotides that codes for no

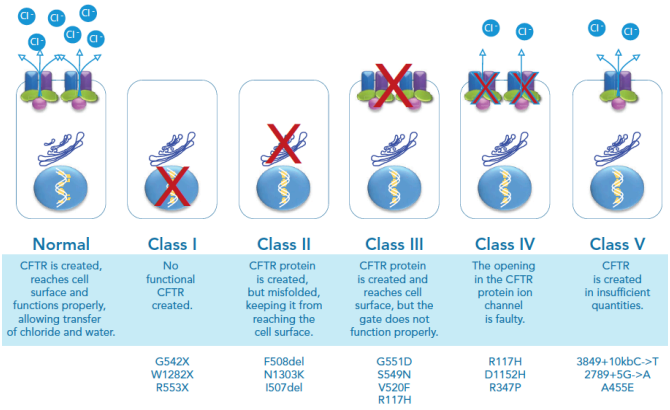

Figure 2. Principal mutations of the CFTR protein.

Phenylalanine at position 508 (Tipirneni K., et al., 2017). As a consequence, the CFTR fails to be transported correctly to the membrane (Cystic Fibrosis Foundation; Patient Registry Reports, © 2016 CFF; De Boeck K. et al., 2013 Welsh M. and Smith A., 1993).

In normal conditions, the CFTR protein represents a transporter member of the ATP-binding cassette (ABC) superfamily, functioning as an ATP-gated pathway for the movement of anions due to a transmembrane electrochemical gradient (Cai Z. et al., 2011).

Thus, a major function of CFTR is to maintain a correct hydration of the airway surface of the liquid layer that cover the mucosal surface.

The mutated CFTR affects the transport of chloride and bicarbonate in secretory epithelial cells of several organs. Furthermore, the altered functioning of CFTR involves a deregulation of the related epithelial sodium channel (ENaC), that normally transfers Na^+ out of the membrane (Cuthbert A.W, 2011). Consequently, the osmotic movement of water across the apical membranes remains altered, resulting in the production of abnormally viscous secretions, which are ineffectively removed by the mucociliary clearance (Kurbatova P. et al., 2015; Robinson M. and Bye P., 2002).

To date, CF represents the most common inherited and life-shortening disorder: the disease manifests as exocrine pancreatic insufficiency, an increase in the sweat of the chloride concentration, male infertility, and airway disease (Cutting G.R., 2014). The predominant cause of morbidity and mortality is therefore represented by the deterioration of the pulmonary functions primarily due by recalcitrant bacterial infections (Guggino W. and Stanton B., 2006).

1.2 CF mucus and rheological behaviour

1.2.1 Molecular composition of airways mucus in CF airways.

Mucus clearance represents a primary innate defence mechanism that protects the airways from hexogen inhaled microparticles. Typically, due to the mucociliary clearance functions, the inhaled particulate is transported from the lower airways to the mouth (mainly microorganisms and particles). Two major components constituted therefore the mucociliary clearance system:

- the cilia system, placed at the apical surface of the airway epithelial cell and called periciliary layer, is constituted by an attached structure of membrane-spanning mucins and muco-polysaccharides, suspended in an aqueous environment;
- the viscoelastic *mucus stratum,* located on top of the periciliary layer, consists mainly of two gel-forming mucin glycoproteins, characterised by a large molecular weight and a high carbohydrate content (Kurbatova P. et al., 2015).

The protein backbone is characterised by the presence of a wide number of tandem repeats (TR), that distinguish mucins from other glycoproteins (Figure 3).

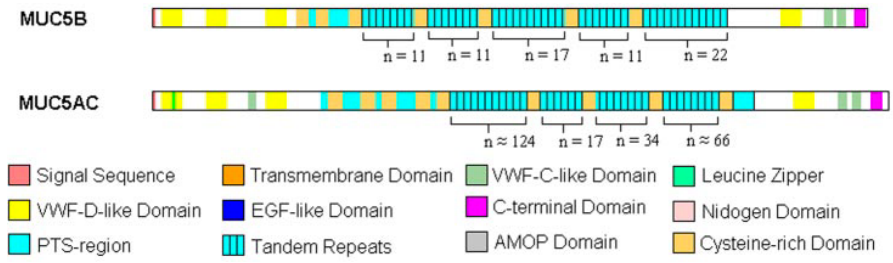

Figure 3. Molecular motifs organisation of MUC protein of some airway mucins.

The classification of mucins involved the MUC protein composition (*apomucin* or *mucin protein core*). Furthermore, mucins could be distinguished in *membrane-tethered* mucins and in *secretory and cysteine rich* mucins (Rose M. and Voynow J., 2006) (Figure 3). The goblet cells commonly express the MUC5AC phenotype, whereas the airway submucosal glands secrete large amount of MUC5B. The central part of mucin is organised in two distinct parts:

- a central region, characterised with TR rich in Ser, Thr, and Pro residues, that holds the O-glycosylation part with the primarily aim to protect the protein core from the proteases action;

- the C terminal and N-terminal regions rich in Cys residues that realise both intra- and inter-molecular disulphide bonds with the aim to create the protein flexibility (Bansil R. and Turner S., 2017). Furthermore, the realisation of the final cross-linked matrix forms a 3D structure that span the mucus gel layer (Lafforgue O. et al., 2017).

In physiological conditions, the epithelial cells secrete mucins by exocytose from pre-stored granules. The mucin polymer is pre-assembled and accumulated with high concentrations of Ca^{2+} and H^{+}, required to prevent its expansion. After secretion, the mucin expansion is then realised by the sequestration of Ca^{2+} and H^{+} ions by HCO_3^{-} (Kunzelmann K. et al., 2017).

In the CF disease, the defective CFTR hinders the HCO_3^{-} transfer across the epithelial membrane and consequently the normal expansion and hydration of the mucin polymers are blocked (Quinton P., 2010; Kunzellman K. et al., 2017). As a consequence, the CF bronchial secretions become highly glycosylated and denser than the non CF secretions, so its accumulation and the consequent inflammation and infections of the tissues lead to a progressive loss of the pulmonary functions (Kreda S. et al., 2012).

In CF, it was also observed that the sputum contains high concentrations of oxidised disulphide cross-links. Moreover, neutrophil oxidants, that are abundant in CF airways, together with an oxidative stress status due to the neutrophilic inflammation, could contribute to the excessive disulphide linkage of mucin in CF mucus (Yuan S., et al., 2015).

1.2.2 Rheological properties of CF mucus.

In physiological conditions, the mucus layer covers and protects the mucosal surfaces due to the presence of mucin glycoproteins, which confer adequate viscoelastic, rheological behaviours. Changes in the physicochemical composition of mucus could affect essential properties such as the mucociliary clearance (Corfield A., 2015; Welsh K. et al., 2017).

Rheology of mucus, investigated for a long time using different sources (*i.e.*, animals, tissues, and organs), represents a key factor in controlling not only the transport of drugs, particles, microorganisms across the mucosa, but also confers adequate properties of hydration and lubrication. Briefly, mucus and mucins exhibit a *non-Newtonian* rheological property; in other words, they are *shear thinning* materials under physiological states, so their viscosity decreases with increasing shear rate. The viscoelastic behaviour depends also by the pH, ionic strength, and degree of dehydration, so the deregulation of the transport pathways causes the pathological conditions, that typical characterise the CF (Bansil R. and Turner S., 2017).

1.3 Microbial infections in CF airways

The CF pulmonary disease is usually related to a progressive deterioration of the pulmonary functions, that commonly lead to morbidity and mortality (Salvatore D. et al., 2016). Indeed, as a consequence of both the inflammatory mediators and both pathogens and their products presence, the viscous pulmonary mucus is secreted in high amounts. Therefore, because of the CF altered viscoelastic behaviour, mucus accumulates in the airways and establishes optimal conditions for the colonization of several bacterial species (Heijerman H. et al., 2009; Kreda S. et al., 2012).

It is well known that CF airway infections (upper and lower airways) are polymicrobial and due primarily to opportunistic and recalcitrant bacteria species (Ciofu O. et al., 2013; Mainz J. et al., 2009). *Pseudomonas aeruginosa*, *Staphylococcus aureus, Haemophilus influenza, Stenotrophomonas maltophilia, Achromobacter xylosoxidans, Bulkholderia* species, together with the fungus *Aspergillus fumigatus,* nontuberculous mycobacteria and even respiratory viruses

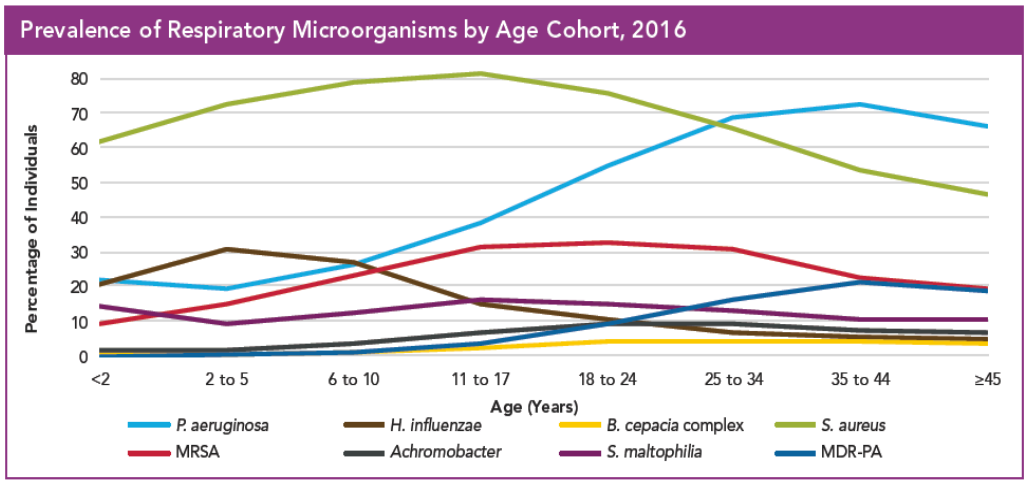

Figure 4. Pathophysiology and management of pulmonary infections in Cystic Fibrosis. Adapted from © CFF 2016.

can supply the basis for the progression of the lung disease (Doring G. et al., 2012; Hauser A.R. et al., 2011).

To date, clinical guidelines provide current best practices to prevent and control exacerbations, and also to reduce exposure to CF pathogens that commonly infect in the health care setting and in everyday life (© 2016 CFF; Figure 4). In February 2017, the World Health Organization (WHO) underlined the importance to achieve a constant dedication concerning the research and development of new antibiotics. Therefore, the aim is to direct the efforts against specific bacteria species that are representing the highest risk to human health, among which *P. aeruginosa* is related to the Priority I class (critical) (Lawie-Davis and Bennett, World Health Organization, 2017; Nicolaou K. and Rigol S., 2017).

1.3.1 CF pulmonary infection by Pseudomonas aeruginosa and Staphylococcus aureus.

Pseudomonas aeruginosa, a Gram-negative bacterium, survives in a simple growth requirement and shows tolerance to a broad range of temperatures. It is characterised by a large metabolic versatility and flexibility, confirmed by the presence of a large number of regulatory genes. Its potential ability to form biofilms, that confers the main adaptive skill, represents an important factor for causing chronic infections, especially the airways of CF patients (Bjarnsholt T. et al., 2013 [(1,2)]).

In the circumstance of the CF pulmonary disease, *P. aeruginosa* is the most studied microorganism. The higher prevalence of infection rises to 80% in patients older than 18, whereas the 50% of young patients (less than 18-year-old) are

infected (© CFF 2016). Initially, infection involve multiple strains and can be intermittent. Afterwards, a single strain prevails for each patient and begins to colonise the lung. at this stage and subsequent, the antibiotic eradication is very difficult (Lipuma J., 2010; Lyczak J. et al., 2002).

Staphylococcus aureus, a Gram-positive pathogen, represents instead the most prevalent organism infecting the respiratory tract of CF children. Often it represents the first specie to be identified hence the transmission of *S. aureus* occurs within households. CF children patients counteract infections prior in the upper airways; after that, infection move to the lung. When identified *S. aureus* alone, in the absence of *P. aeruginosa,* a milder pulmonary disease could be defined. Otherwise, when infection is associated with *P. aeruginosa*, patients report a poorer survival prospect.

As reported also in the Dorothy Andersen's seminal report, *S. aureus* was a major cause of death in (young) CF patients, in the pre-antibiotic era (Andersen D., 1938). Subsequently, after the routinely usage of antibiotics (against *S. aureus)*, there was during time a change in the predominant species. Thus, *P. aeruginosa* become the major cause of lung worsening, while *S. aureus* remains the second most prevalent organism in CF adults. (Ahlgren H. et al., 2015; Archer N. et al., 2011; Armbruster C. et al., 2016; Wong J. et al., 2013).

1.3.2 The biofilm issue.

Bacteria can grow in two different ways: the first is a planktonic, or swimming phenotype, in which the cells are "free floating" in the culture medium.

Nonetheless, as confirmed by DNA-sequencing methods, less than 0.1% of the total microbial biomass on Earth is in the planktonic phenotype, whereas the majority is organised in aggregates that are enclosed in a biofilm matrix (Bjarnsholt T. et al, 2013 [(1)]).

The second way involves the ability of the sessile bacterial cells, both Gram-

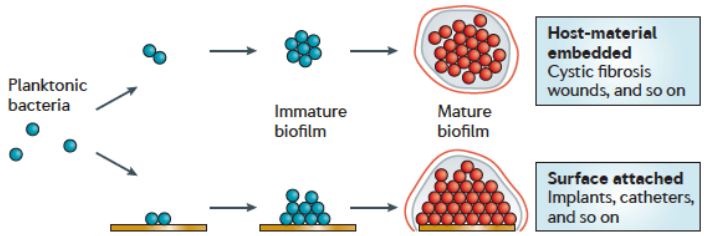

Figure 5. Biofilm development from a planktonic growth.

positive and Gram-negative, to aggregate in a secreted polymeric matrix. When bacteria form aggregates by attaching to a surface, or to each other, the development of the biofilm starts (Figure 5). In an optimal environment, biofilm becomes mature and able to protect the dormant, very slow growing, or dead cells within. This mode of growth represents an adaptation mechanism to survive in a hostile environment (Archer N. et al., 2011).

The chemical constitution of biofilm comprises mainly polysaccharides, proteins, and extracellular DNA. Three kinds of polysaccharides have been recognised in *P. aeruginosa* biofilm: *Psl*, *Pel*, and alginate. *Psl* was identified in the 2004: it operates as a scaffold in PAO1 strain for the biofilm development. Failure to produce *Pel* or *Psl* impairs therefore the biofilm formation *in vitro*. *Psl* and *Pel* are also normally produced by non-mucoid strains.

Alginate is often produced by *P. aeruginosa* CF clinical respiratory isolates. The typical mucoid phenotype is due to the overproduction of this polysaccharide and plays an important role in CF lung colonisation. However, it is not absolutely

required during the formation of non-mucoid biofilm *in vitro* (Visaggio D. et al., 2015; Wei Q. and Ma L., 2013).

Another important component of biofilm is constituted by the extracellular DNA (eDNA) with several functions, evaluated firstly and not only in *P. aeruginosa*, but also identified in *S. aureus* biofilm components.

Anyway, bacterial biofilm confers resistance to antimicrobial agents (10 to 1000 fold) (Reffuveille F. et al., 2014), and these findings could be linked to the worsening conditions of the CF lung. Therefore, to control the development of the

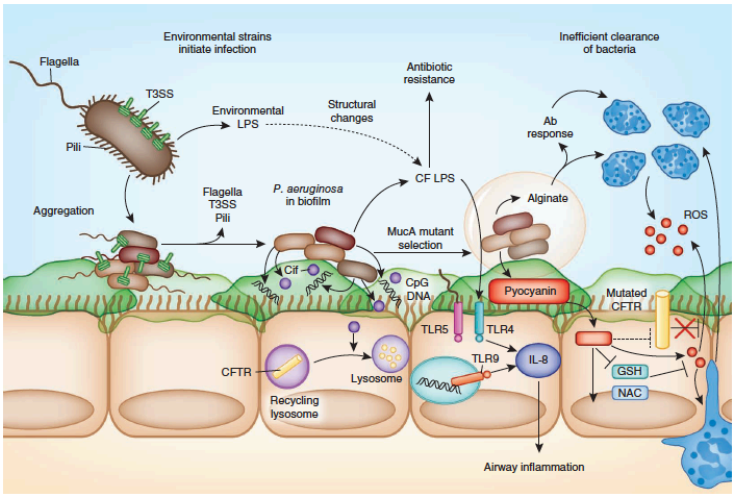

Figure 6. Adaptation of the inhaled bacteria to the Cystic Fibrosis airways.

recalcitrant infections, a better understanding of the chromosomal and molecular mechanisms of biofilm formation may provide additional resources to discover new molecules able to prevent the formation of the biofilm matrix (Figure 6) (Wei Q. and Ma L., 2013). Another strategy adopted by *P. aeruginosa* to survive within the host is represented by the multidrug tolerance ability.

Persister bacteria in biofilm seems to be responsible for the worsening nature of the recalcitrant infections, because these cells remain viable and repopulate when the level of the antibiotic slow down (Wood T. et al., 2013).

The opportunistic pathogens show numerous and intrinsic mechanisms of resistance, including enzymatic arsenal to survive in presence of antibacterial and the expression of multidrug efflux pumps (Nadal Jimenez P. et al., 2012; Nicolaou K. and Rigol, S., 2017). Moreover, the *quorum sensing* (QS) signalling in *P. aeruginosa* activate a mechanism that permit the communication among bacteria in response to changes in population density, involving the use of at least three signal molecules. These three QS systems are primarily involved in the regulation of virulence factors as well as cellular processes such as chemotaxis and biofilm formation (Bjarnsholt T. et al., 2010).

1.4 Symptomatic treatments of CF lung infections – antibiotic therapies

Depending on the stage of colonisation (planktonic or biofilm mode of growth) and the phase of infection (acute or chronic), different situations can be distinguished for the use of antimicrobial infections. In each case patients require different therapeutic approaches. The utilisation of proper antimicrobial therapies should help to reduce bacterial population and to be fast-acting to avoid the selection of resistant mutant bacteria, and prevent the phenotypic tolerance (Canton R. et al., 2005).

Different administration routes (oral, inhalation, intramuscular, and intravenous)

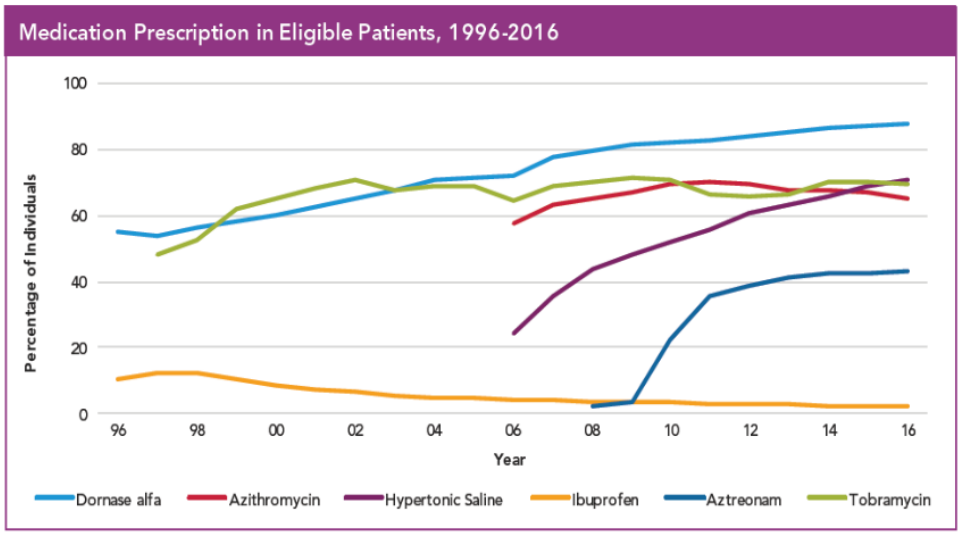

Figure 7. Trends in the prescription of pulmonary medications recommended for chronic use by the CF foundation pulmonary guideline committee.

are available for the antibiotic treatment (Figure 7). However, for the CF pulmonary disease, the antibiotic delivery to the lung represents nowadays a smart option (Geller D. et al., 2011). Indeed, it is possible to achieve the therapeutic effect by administering a relatively high dose (the labelled doses ranging from 75 to 300 mg) directly to the lung.

A summary of the utmost importance antimicrobial agents and formulations which are being currently used to treat *P. aeruginosa* infections in CF patients is discussed.

The first antibiotic solution, produced for inhaled therapy, concerns the aminoglycoside Tobramycin. Nonetheless, the inhaled solutions for nebulisation, that have been overused for approximately 15 years (Bramitob®, Tobi®, and Tymbrineb®), do not represent an optimal medical treatment (Nokhodchi A. and Martin G., 2015), especially towards the emerging MDR bacteria. Tobramycin DPI

is also available on market, (*i.e.,* Tobi® PodHaler™) with the main aim to simplify the long-lasting administration concerning the antibiotic management in CF.

Recently, the European Medicines Agency (EMA) approved the sodium colistimethate (polymyxin E), a polymyxin antibiotic, marketed in a powder for a solution for nebulisation Promixin® and in Dry Powder for Inhalation (DPI), Colobreathe®. Colistin is a 50-year old cationic polypeptide antibiotic that is currently used in last-line CF clinical practice. However, in recent years worldwide have emerged reports of colistine resistance (Lee S. et al., 2016).

Ultimately, the inhaled antibiotics approved by the Food and Drug Administration (United States) and EMA are summarised below:

- Aztreonam inhalation solution (Cayston®);
- Colistimethate sodium inhalation solution (Colistin) and inhalation powder (Colobreathe®) only approved by EMA;
- Levofloxacin nebulizer solution (Quinsair®);
- Tobramycin inhalation solution (TOBI®) and inhalation powder (TOBI® Podhaler™) (Hamed K. and Debonnett L., 2017).

Currently in clinical practice, the administration of inhaled colistine, tobramycin or aztreonam with or without oral ciprofloxacin are suggested to manage the initial infections. During mild moderate exacerbations, oral ciprofloxacin can be administered. Severe exacerbations must be treated with intravenous combination therapy (β-lactam with aminoglycoside or fluoroquinolone). When infections become chronic, a 28-day on-off regimen with tobramycin or aztreonam or constant administration of colistine the treatment is recommended (Canton R. et al., 2015).

1.4.1 Promising antibiotic and anti-biofilm approaches.

Old antibiotics has been basically developed on narrow chemical scaffold sources and only few innovations since 1980's were carried out (Gonzales-Lamothe R. et al., 2009). Contemporary researches described that "no new antibiotic lead" are available; on the other hand, the resistance skill by MDR bacteria increases constantly (Wright Gerard D., 2017). The results from AstraZeneca's antibacterial discovery, between 2001 and 2010, illustrated that the identification of new hit (a compound with a lack of activity) was not the principal efforts in their drugs discovery programmes (Brown E. and Wright G., 2016; Tommasi R. et al., 2015). Nonetheless, because of the relatively small number of CF case, the costs of carrying out clinical trials for new antibiotics have always been considered particularly expensive (IMI 9th Call for proposals: focus on frailty, use of social

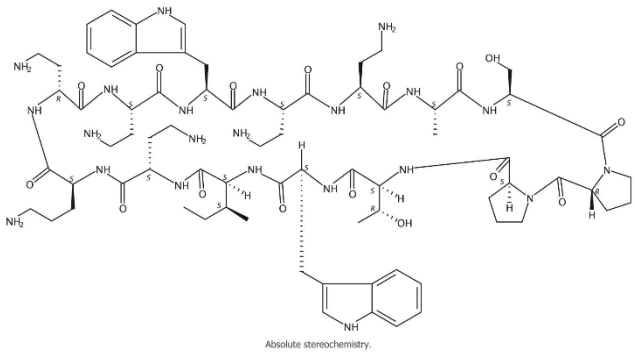

Figure 8. Murepavadin (POL 7080).

media to monitor drug safety and antibiotic development, IMI).

Fortunately, several agencies worldwide such as the Infectious Disease Society of America (ISDA), the Centers for Disease Control (CDC), and the World Health Organization (WHO) have pointed out this issue and the simultaneous problem regarding the increase of the antibiotic resistance. Thus, the European Union (EU) and the Innovative Medicine Initiative (IMI) realised the project ND4BB (New Drug

for Bad Bugs). The aim of these efforts consist into fund a public-private partnership, so that the development of the new antibiotic drug discovery could be accelerated (Bush K. and Page M., 2017). Both Polyphor and the IMI are supporting the development of an inhaled formulation of Murepavadin (Figure 8; POL7080 in Phase 2 clinical studies, specifically for CF lung infections).

Its pharmacologic action consists in an outer membrane protein targeting antibiotic (OMPTA) that could help to fight the battle towards recalcitrant CF *P. aeruginosa* lung infections (Ferrandez and Condemine, 2008; Luther A. et al., 2017; "Polyphor Gets New Funding to Develop CF Inhaled Antibiotic Murepavadin," 2017).

In other recent published papers, other drug candidates are reported: the monosulfactam (β-lactam) BAL 30072 (Figure 9) showed promising activity against contemporary Gram-negative bacteria, including MDR *P. aeruginosa* (Bush K. and Page M., 2017; Landman D. et al., 2014). However, an important side effect of BAL 30072 was also observed: the increase transaminase activities in healthy subjects in multiple dose clinical studies (Paech F. et al., 2017).

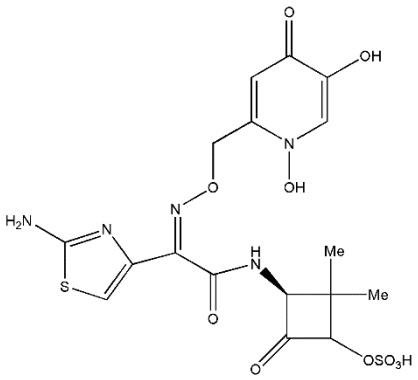

Figure 9. BAL 30072.

Other new drug candidates are reported elsewhere, peptides and enzymatic agent could act as anti-biofilm agents or have an effect on prevention, destabilisation or

inhibition of the biofilm structures (de la Fuente-Nunez C. et al., 2014; Hwang I., et al., 2017; Reffuveille F., 2014).

Chapter 2

DRUG DELIVERY TO THE LUNG

2.1 Anatomy and physiology of the lung

To fully understand the drug deposition and distribution in the lungs after the inhalation, it is important to consider several properties of the respiratory system. A very useful method is to categorise the respiratory tract into two principal parts:

- the upper respiratory tract, that comprises the nose, the nasal cavities, and pharynx;
- the lower respiratory tract that consisting in the larynx, trachea, and alveoli

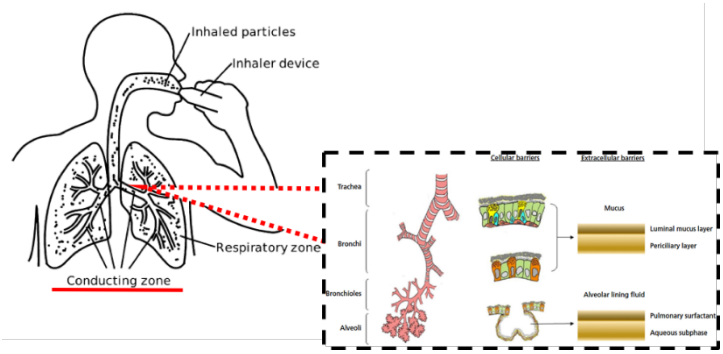

Figure 10. The two functional zones of the airways.

(Hastedt J. et al., 2015).

The airways can also be divided in two distinct functional airways zones, according to the Weibel model:

- the conducting zone (generations 0–16) composed of the trachea, bronchi, bronchioles and terminal bronchioles, which are responsible for conducting air to the respiratory zone;
- the respiratory zone consists of respiratory bronchioles, alveolar duct and the gas exchange region (alveolar sacs) (Weibel E., 1963).

To discriminate among the local or systemic therapies, the pulmonary route permit to achieve the conducting zone (bronchi and bronchioles) for the treatment of

obstructive respiratory disease (local-acting drugs), or to reach the enormous surface area of the respiratory zone (alveoli) for a systemic effect.

2.2 Background of pulmonary drug delivery

The lung represents an alternative route of drug delivery, since it is possible to improve the concentration of the drug locally, minimising at the same time the required dose to exert the therapeutic action. Compared with systemic exposure, the maximal concentration of an inhaled drug is higher in the lung tissue after a local administration. This delivery method could help to retain the drug for the required time, to prevent potentially toxic therapeutics from entering the blood stream, and therefore to limit possible adverse effects towards the other healthy organs. Moreover, drug-metabolising enzymes are in smaller amounts in the lungs compared to liver and gastro intestinal tract.

For these reasons, the pulmonary route has been used to deliver protein, peptides, chemotherapeutics, interferon, anti-trypsin, protease inhibitors, vaccines and so on

Figure 11. Various indications for which biotherapeutics have been evaluated using the inhalation route.

(Muralidharan P. et al., 2015; Pulmonary & Nasal Delivery – OndrugDelivery, Issue 80, 2017) (Figure 11).

The inhalation of drug is often used for the management of several local diseases, such as the chronic obstructive pulmonary disease (COPD; 329 million people affected globally in 2010), asthma (about 235–330 million asthmatics around the world in 2011) and CF lung disease. Hence, for protracted administration, the pulmonary drug delivery represents a valid option, since it offers several advantages *i.e.*, self-administration, a rapid onset, improved patient compliance (Liang Z. et al., 2015).

Current inhaled drugs for the management of lung diseases on the market comprise: short-acting and long-acting β-2 agonists, anticholinergics agents, corticosteroids. Moreover, antisense and oligonucleotides are reported in Phase 2 and Preclinical study, respectively.

Specifically, for CF pulmonary disease, antibiotics (tobramycin, aztreonam, and colistimethate sodium), mucus mobiliser (Dornase alpha®), hypertonic saline to restore the airways surface liquid are currently marketed. Additionally, anti-protease, and antibiotics for multi resistant *Staphylococcus aureus* (MRSA) treatment are reported in Phase 2. (Wang Y. et al., 2014).

2.3 Drug products for inhalation

According to the Pharmacopoeia's Monograph (Eur. Phar. 8th, monograph 0671), the preparations for inhalation (*Inhalanda*) are defined as:

"*liquid or solid preparations to be administered to the lungs as vapour or aerosol, to obtain a local or systemic effect. They contain one or more active*

ingredients that can be dissolved or dispersed in a suitable vehicle. (…) Inhalation preparations are supplied in multidose or in a single dose container.

The preparations to be administered as aerosols (dispersions of solid or liquid particles in a gas) are administered with one of the following devices:

- *nebulizer,*
- *a pressurized metered-dose inhaler (pMDI) or powder inhaler (DPI)".*

The *nebulizer* creates a spray from an aqueous based drug, solution or suspension, by air jet or ultrasonic instruments. It is typically used to manage acute and short-lasting diseases and for deliver the API over multiple breaths to children, elderly and critically ill patients. Several drawbacks characterise these products: *e.g.*, jet nebulisers operate by a noisy compressor. Together with ultrasonic nebulisers, both kinds of products require a power source, limiting their portability. The administration of each unit-dose is therefore time-consuming. Also, regular cleaning and disinfection procedure of the system's pieces are required after each administration manoeuvre. Thus, the time needed for each daily administration become particularly critical for the CF pulmonary management.

The *pMDIs* represent the most widely used products, mainly for the pulmonary delivery of highly potent drugs. Thus, only small doses of less than a milligram can be delivered with a pMDI. Furthermore, the administration requires coordination between the inspiration and the dispensing procedure of the medication. (Claus S. et al., 2014).

2.3.1 Dry Powder for Inhalation (DPI).

The *DPI* product (Figure 12) was originally developed to avoid the coordination

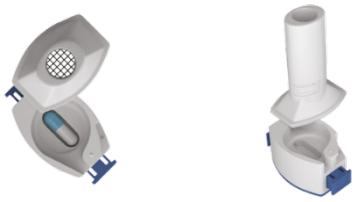

Figure 12. A DPI product (device and capsule) from Plastiape® model RS01.

required to dispense the dose and the breath of the patient, and also to avoid the usage of chlorofluorocarbons (CFC) propellant, typical of the pMDI formulations. Actually, an antibiotic formulated as a DPI overcomes some of the drawbacks related to the nebulisation procedure. It could encompass several important factors which contributes to the enhancement of the compliance, such as the reduction of the delivery time, the daily treatment time, the drug dose load in a single dosage form, and the daily dose (VanDevanter D. and Geller D., 2011). An interesting information is reported in the literature regarding the marketed antibiotic product, routinely recommended for CF pulmonary infections: the tobramycin formulations for inhalation. As discussed, various clinical trials validated a higher patient satisfaction when using the powder formulation, suggesting that the suitability, as well as a lower treatment burden related to the administration, may result in an improved compliance. Moreover, the daily dose in dry powder form becomes consistently reduced (128 mg = 4×28 mg) *vs* 300 mg /5 ml of tobramycin in dry powder and liquid formulations, respectively), reporting a similar systemic exposure and no evidence of serum accumulation of the drug after the dry powders administration was revealed (Hamed K. and Debonnett L., 2017).

Also, to convert the drug in a dry powder form is easy to realise, and also permits better physicochemical stability of the API (De Boer A. et al., 2017; Hoppentocht M. et al., 2013).

Most devices are breath actuated, so the emission of the powder medication is coordinated with the patient's inhalation (Derek I. and Jesse Z., 2008). There are two types of dry powder inhalers (DPIs): passive (breath actuated) devices, and active devices. With passive devices, the energy for dispersion of the dry powder is generated by the patient's inspiratory manoeuvre. In contrast, active devices minimise inspiratory effort by using a compressed gas to suspend the powder in the air flux. The active devices have also recently been mentioned to the third generation DPIs (Yang M. et al., 2014).

Moreover, DPIs could be further distinguished in four typologies: a very common type of DPI that requires to load the single-unit dose (capsule) containing the drug in a dry-powder form into the device before use. The device is also equipped with pins that pierce the capsule to allow the aerosolisation of the powder (Figure 12). The single-unit dose (disposable) DPI contains a pre-metered amount of a single dose, that is wasted after use. Other common devices are represented by the multi-unit dose DPI (pre-metered unit replaceable set) that dispense individual doses from a pre-metered replaceable container (*i.e.*, blisters, disks, dimples, or tubes). Multiple dose DPI (reservoirs) that encloses a bulk amount of drug powder in the device. A built mechanism meter each dose from the bulk (Buttini F. et al., 2012).

2.4 Factors affecting the aerosol deposition in the lung

The inhalation products, especially if formulated as a dry powder for inhalation, have to guarantee both the right dose emission and the drug deposition in the desired zone of the lungs. Particles intended to be administered by pulmonary route are comprise within the dimensional classes of *coarse particles* (> 5μm), *fine particles* (between 3 and 5 μm), and *ultrafine particles* (between 3 and 0.5 μm). For an optimal deposition, and a more specific targeting in the desired region of the lungs, a narrow size distribution, or monodisperse aerosol, is required. Other factors that could affect the deposition include the diseased state and breathing patterns of the individual (breathing rate, lung volume, and respiration volume) (Muralidharan P. et al., 2015).

In the engineering of dry powder microparticles, particle size and size distribution,

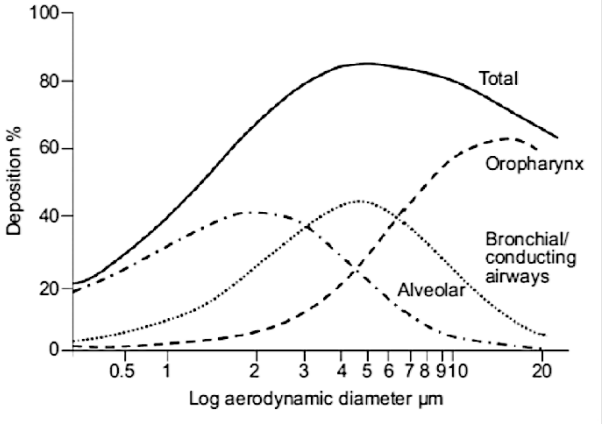

Figure 13. Relationship between the aerodynamic diameter and lung deposition.

particle morphology (*e.g.*, spherical particles, fibres), surface morphology (*e.g.*, smooth, wrinkled), have been identified as critical parameters, with the final aim to optimise site-specific lung targeting. Moreover, the hygroscopicity, electrical charge, and density influence the aerodynamic behaviour of the microparticles (Muralidharan P. et al., 2015).

The aerodynamic diameter (d_{AE}; Equation 1) defines the behaviour of the particles when dispersed in the airstream. It is generally accepted that particles with a d_{AE} ranging between 3 and 5 μm tend to deposit in the conducting zone of the airways (trachea-bronchial region), whereas smaller particles, ranging from 0.5 to 3 μm, are better delivered in the respiratory zone (alveolar). Sub-micron sized particles less than 0.5 μm are generally exhaled during a normal breathing (Laube B. et al., 2011) (Figure 13).

The d_{AE} is defined as a spherical equivalent diameter that derives from the equivalence between the inhaled particle and a sphere of unit density undergoing

$$d_{AE} = d_v \sqrt{\frac{\rho}{\chi \rho_0}}$$

Equation 1

the sedimentation at the same rate. d_v represents the volume-equivalent diameter, ρ is the particle density and χ is the dynamic shape factor. χ is a very important aerodynamic parameter, that takes into account the non-sphericity of the microparticles, becoming equal to 1 for a sphere, and greater than 1 for irregular-shaped microparticles. Thus, d_{AE} values can be reduced by:

- Decreasing the volume-equivalent particle diameter, d_v;
- Reducing the particle density, ρ;
- Increasing the particle dynamic shape factor, χ.

2.4.1 Excipients used for DPI products.

To disperse the powder, a DPI product combines both the technological properties of the microparticles (*e.g.*, size distribution, surface modification, density) and the design of the device. However, due to the small size, the API microparticles show often adhesive and cohesive forces, and therefore a failure of the aerodynamic behaviour. The addition of inert ingredients aids to enhance several properties of the resulting powders (Table 1).

Table 1. List of accepted or interesting additives for DPI formulations (adapted from Pilcer et al., 2010).

Excipient	Description	Status
Sugars Lattose, Glucose[1], Mannitol[2], Threalose[3]	Coarse/fine carrier	Approved and commonly used ([1]Bronchodual®;[2]Exubera®). [3]Promising alternative.
Hydrophobic additives Mg stearate	Protection for drug moisture	Approved (SkyeProtect™).
Lipids DPPC, DSPC, DMPC, cholesterol	Used in liposomes, matrix, coating	Biocompatible and biodegradable; very interesting excipients.
Amino acids Leucine, trileucine	Improved aerosol efficiency	Endogenous substance but no data about lung toxicity.
Surfactants Poloxamer[1], Bile salts[2]	Production of light and porous particles	[1]May be not proinflammatory at low dose; [2]Endogenous substances. May be accepted at low dose (2-5%w/w).
Absorption enhancers [1]Hydroxypropilated-β-CD; natural-γCD [2]Bile Salts [3]Chitosan, trimethylchitosan	Apsorption for proteins and peptides	[1]Promising results. [2]Promising results but toxic in chronic use. [3]Proinflammatory effect observed.
Biodegradable polymers PLGA	Used in sustained release formulations	Immunogenicity effect observed.

To increase the flowability, the addition of safe *amino acids* (*e.g.*, Arginine, Histidine, Leucine, Lysine, Proline, Threonine) represents a further strategy. As reported, the co-spray-dried microparticles API with amino acid permitted to improve the drug aerosolisation (Prota L. et al., 2011).

The most commonly used excipient in the marketed DPI is *lactose*: it is safe, stable, and less hygroscopic than other sugars (*e.g.*, mannitol, glucose monohydrate, trehalose, dextrose, maltose, sorbitol, maltitol, and xylitol). Moreover, lactose is cheap and readily available, but also shows satisfactory flow properties when micronised. Indeed, it may be used in a broad size range (50 - 200 μm) and

operates as a *carrier* when mixed with the API microparticles. During the inhalation manoeuvre, and also due to the different aerodynamic diameter of each constituent, a blended formulation permits that the particles of both carrier and the API are prone to separate. The carrier, due to the large size, impacts with the oropharynx and then cleared, whereas the API microparticles continue to follow the flow of the airstream, to deposit in the lower airways (Pilcer G. et al., 2010). However, lactose is a reducing sugar, thus it may show incompatibility with drugs that have primary amine moieties (Telko M. and Hickey A., 2005).

Beside this, *lipidic excipients* are also available in the marketed products (*e.g.*, Qvar® beclomethasone dipropionate, a poorly water-soluble drug formulated for the pulmonary route). Some authors illustrated that the addition of lipids permits to enhance some technological properties, allowing for the avoidance of the agglomeration of the powders.

Furthermore, a formulation of the antibiotic tobramycin coated with lipids (9:1 cholesterol: distearoyl phosphatidylcholine) showed a modified surface of the microparticles. This approach permitted to enhance the Fine Particle Fraction (FPF), one of the aerodynamic parameters, and refers to the respirable fraction constituted of powders with $d_{AE} < 5\ \mu$m. Compared to the uncoated antibiotic batch, only the lipid-coated tobramycin was associated with the higher respirable fraction (FPF 36% *vs.* 68%, respectively).

Lipidic excipients are the same as the constituents of the lung surfactant (like phosphatidylcholine), so they can also be metabolised and cleared. Nonetheless, the safety of excipients for the pulmonary drug delivery is often accompanied by undesired oxidative degradation processes (Nokhodchi A. and Martin G., 2015; Pilcer G. et al., 2010).

2.5 Mechanisms of particle deposition in the airways

The deposition of a drug administered by the pulmonary route can occur via *impaction, sedimentation, interception*, or *diffusion* mechanisms.

Impaction is the prevailing deposition mechanism for particles (or droplets) larger than 5 μm; the mechanism is flow-dependent and is determined by the d_{AE}. Large particles with high velocity may be unable to change direction with the inspired air, so they impact passively onto the mucosa. This mechanism is common for both the upper and the tracheobronchial region deposition, where the air velocity is high and the airflow is turbulent. The deposition probability by impaction *p(I)* in cylindrical airways is calculated as shown in Equation 2, in which *St* refers to *Stoke number* (Equation 3):

$$p(I) = 1 - \frac{2}{\pi}\cos^{-1}(\theta\ St) + \frac{1}{\pi}\sin[2\cos^{-1}(\theta\ St)]$$

Equation 2

$$St(Stoke\ number) = \frac{\rho v d^2}{18\eta D}$$

Equation 3

where θ represents the branching angle, ρ is the density of the particle, η is the viscosity of fluid, ν is the velocity of the particle, *D* is the airway diameter, and *d* is the particle diameter.

Microparticles in the size-range of 0.5 – 5 μm may avoid impaction and deposit in the lower bronchial region where the airflow is slower with a mechanism of *sedimentation*. The rate of deposition increases with the particle size and decreases with the flow rate, so the sedimentation of the particles is governed by

the gravitational force. The deposition probability by sedimentation *P(s)* in cylindrical airways is calculated as shown in the Equation 4:

$$P(s) = 1 - e^{\frac{4gC\rho d^2 L \cos\Phi}{9\pi\eta R v}}$$

Equation 4

where *g* is the gravity acceleration, Φ is the angle relative to gravity, *L* is the tube length, ρ is the density of the particle, *C* is a correction factor, *d* is the radius of the particle, *R* is the radius of the airways, and η is the viscosity of fluid.

The most effective deposition mechanism for particles characterised by acicular shapes (fibres) is *interception*. Deposition may occur because the value of the d_{AE} is smaller relative to their size, so they usually can deposit up to the respiratory zone.

Sub-micron sized particles (< 3 μm) deposit by Brownian motion, according to the mechanism of *diffusion*. This mechanism is governed by the geometric rather than the aerodynamic diameter. The deposition probability by diffusion *P(d)* in cylindrical airways is calculated as reported in the Equation 5:

$$P(d) = \sqrt{\left(\frac{2KTC}{3\pi\eta d} \cdot \frac{1}{D}\right)}$$

Equation 5

where *D* is the airway diameter, *k* is the Boltzmann constant, *T* is the temperature (°K), η is the gas viscosity, and *d* is the particle diameter (Nokhodky A. and Martin G., 2015).

2.6 Relevance of solubility and dissolution process of a solid drug in the lung

Solubility is a property of a solute (solid, liquid, or gaseous) to form a homogeneous solution in a solid, liquid, or a gaseous medium. The solubility of a substance

Table 2.

USP and BP Solubility Criteria.

Descriptive Term	Parts of solvent required for 1 part of solute
Very soluble	< 1
Freely soluble	1 – 10
Soluble	10 – 30
Sparingly soluble	30 – 100
Slightly soluble	100 – 1000
Very slightly soluble	1000 – 10 000
Practically insoluble	> 10 000

fundamentally depends on the solvent used as well as on temperature and pressure. The amount of solubility of a substance in a specific solvent is measured as the saturation concentration where adding more solute does not increase its concentration in the solution, forming a precipitate (Savjani K. et al., 2012). This amount can be expressed in precise terms (*e.g.*, mg/ml, g/L) or in general terms, as reported by both the British and the United States Pharmacopoeias (Table 2) (Smith B., 2015).

Nonetheless, dissolution represents a process by which a solid substance interacts with a solvent to yield a solution during time. The process is controlled by the affinity between the solid substance and the solvent.

In general terms, the dissolution rate can be expressed by the Noyes – Whitney equation (Equation 6; Table 3), thus, once inhaled and deposited in the lung, the

microparticles enter in contact with the fluid covering the lung epithelium, and the drug dissolve in accordance with the Equation 6:

$$\frac{dm}{dt} = \frac{A \times D \times (C_s - C_t)}{h}$$

Equation 6

The dissolution rate of a drug in the lung is difficult to assess *in vivo*, so a standardised *in vitro* dissolution method is in itself very difficult to design because the lung has several unique qualities, such as the small amount of aqueous fluids that cover the surface, the presence of endogenous surfactant. Also, the dissolution rate of poorly water-soluble drugs depends on both the wettability and solubility of the drug in the present fluids. (Riley T. et al., 2012).

Table 3.
List of variables and coefficients reported in the Noyes-Whitney equation.

dm/dt	Dissolution rate of the solid drug
A	Surface Area of the drug solid in contact with the dissolution medium
D	Dissolution coefficient
h	Diffusion layer thickness
Cs	Drug saturation solubility at the drug solid surface
Ct	Drug concentration at time t in the well stirred bulk

Ct is estimated to be negligible under the so-called SINK conditions (*i.e.,* when $Ct \ll Cs$).

2.6.1 Formulation strategy to enhance the poorly water soluble drug for the pulmonary route.

As for oral administration, for the pulmonary route dissolved drug particles can act with the local pharmacological target. On the contrary, undissolved drugs are destined to be cleared or aggregated by the mucociliary escalator in the conducting

zone, or phagocytised by alveolar macrophage, if the deposition involves the respiratory region. This issue remains essential to overcome, since a drug has to be dissolved to exert its pharmacological action. To reach this aim, the development of a proper formulation could be helpful to make a poorly water-soluble drug able to better interact and dissolve in the lung secretions enhancing, therefore, its dissolution rate.

To improve several properties of a micron-sized drug, as well as the wettability and the dissolution properties of the poorly soluble APIs, simple strategies could be adopted, *e.g.*, salt form selection, pH adjustment, particle size reduction. Furthermore, the addition of inert ingredients, or excipients, could be a support to enhance the chemical or pharmaceutical properties (*e.g.*, solubility/dissolution properties), as well as to preserve better the physicochemical stability of the APIs. However, for the pulmonary administration the choice of *generally recognised as safe* excipients to improve the solubility and, therefore, the efficacy of poorly-water soluble drugs, is very restricted, since only a few amount is approved by the Food and Drug Administration (Table 3).

In sum, the use of excipients in a formulation could enhance the wettability and the dissolution process of the poorly water-soluble drugs even more for the pulmonary administration; on the other hand, the absence of excipients in a DPI formulation could:

- permit to increase the drug load in each unit-dose, especially for low potency drugs (*i.e.,* some antibiotics);
- avoids the problem of long and costly studies and the risk of rejection by regulatory authorities (Pilcer G. et al., 2013; 2010; 2009).

2.6.2 Dissolution test for inhaled products.

To measure the dissolution behaviour of inhaled powders, no standardised methods are reported in Eur. Pharm. 8th. The development of an *in vitro* dissolution assay, for the orally inhaled drug products, requires to define several aspects, *i.e.*, the dissolution apparatus type and the modifications adopted. Also, because of there is no standard and representative dissolution media, it is necessary to specify the volume and the composition of them, especially to test poorly soluble drugs (*i.e.,* the presence of additive like polysorbate or surfactants in the dissolution medium) (Riley T. et al., 2012).

Nonetheless, dissolution tests for DPI medications have been reported in the literature almost for ten years, to characterise the *in vitro* behaviour of particles within the formulation development, but also to investigate about the API bioequivalence or the behaviour of the excipient used (Velaga S. et al., 2017).

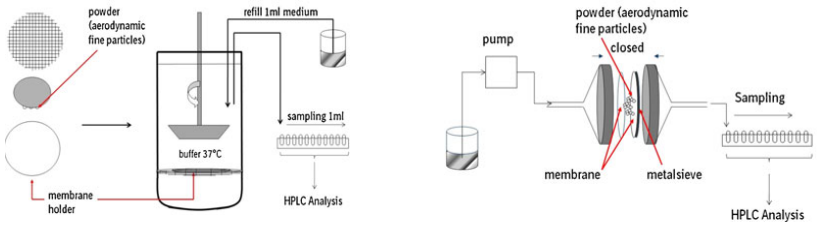

Figure 14. Schematic representations of the paddle method for the dissolution test (Apparatus 2; Eur. Pharm. 20903, left). The flow-through cell (Apparatus 4; Eur. Pharm. 20903, right) (adapted from May et al., 2012).

Different techniques to assess the dissolution process are reported in the literature. The basket method of dissolution and the paddle method (Apparatus 1 and 2, respectively; Eur. Pharm. 8th 20903) are suitable to define a proper technique for inhaled products in dry powder form. The flow-through cell (Apparatus 4; Eur.

Pharm. 8th 20903) was also used to develop a further dissolution test for the inhaled formulations (Figure 14) (May S. et al., 2012).

Several studies reported that the paddle apparatus (Apparatus 2), equipped with the membrane holder, shows the best discrimination power with optimal reproducibility between formulations (May S. et al., 2012). It's important to evidence that without the membrane holder, in fact, the speed rotation of the paddle method (Apparatus 2) may cause an accumulation of the powder that aggregates to form a conic shape at the bottom of the vessel.

To obtain further evaluations of the dissolution process for the dry powder for inhalation, several authors demonstrated that the diffusion-controlled systems (Franz cell, Transwell® units, dyalisis) can be selected to mimic the small amount of fluids lining the lung (ensuring the fluid-restricted dissolution conditions) and the diffusion of the drug in a controlled air-liquid interface (Nokhodky A. and Martin G., 2015). In contrast, the paddle apparatus is based on the fact that the membrane is completely immersed and the dissolution process results flow-rate controlled. Together with the flow-through cell, these dissolution methods have no air-liquid interface compared with the Franz cell method, in which the dissolution process is diffusion-controlled.

Furthermore, a Franz cell apparatus (Figure 15) could be properly used in a modified configuration to study the dissolution process of a drug both through a CF sputum sample or artificial mucus models (Russo P. et al., 2013).

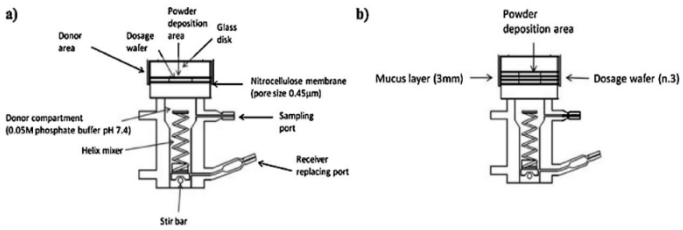

Figure 15. The Franz cell apparatus used in permeation studies: (a) standard configuration with one dosage wafer housing the drug formulation; (b) modified configuration with three dosage wafers housing mucus and drug formulation (adapted from Russo et al., 2013).

2.7 Particle engineering to produce microparticles

Spray drying represents a widely applied technology consisting in the preparation of a feed solution of the drug, dissolved or dispersed generally in a hydro-alcoholic vehicle (both alone or in presence of excipients). Consequently, the liquid feed is atomized (sprayed) in the drying chamber and the solid content within is converted into a dried particulate (Figure 16).

The principal advantages of the technique are:

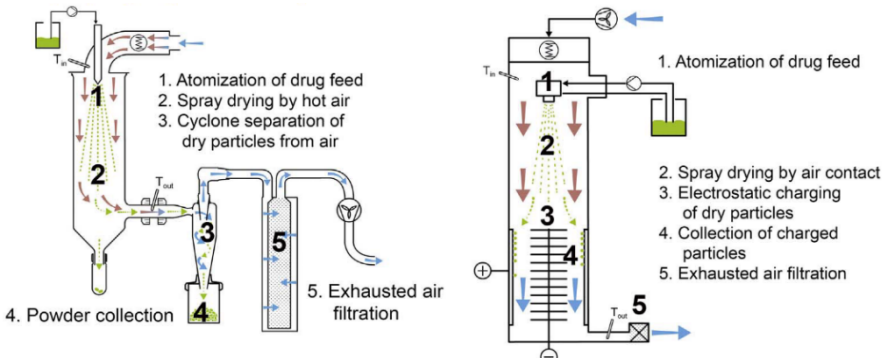

Figure 16. Operation schemes of Mini (left) and Nano (right) Spray Driers.

- processing of both solutions and suspension, at different total solid concentrations;
- multiple options with respect to the liquid feed composition;
- the process parameters, such as feed rate and evaporation temperature, according to the drug to process are easily regulated (*i.e.*, lower temperature for temperature-sensitive drugs) (Tiozzo Fasiolo L. et al., 2017).

2.7.1 Spray drying method to obtain dry powders for inhalation.

Moreover, this method permits to obtain fine particles with a more homogeneous particle size distribution, which comprise the respirable fraction (< 5 μm). For this reason, this technique has been used to prepare dry powders of antibiotics, anti-inflammatory and insulin powders for a pulmonary administration (Belotti S. et al., 2015).

Indeed, specific variables of the spray drying process can be controlled by modification of several parameters besides the drying temperature: the composition of the solvent, the solute concentration, the feed rate. This contributes to achieve pre-determined characteristics of the resulting powders, *e.g.*, habit (size, morphology, shape), and density.

Specifically, the spray drying technology permits to decrease both density and size of the microparticles (Chow A. et al., 2007). Moreover, to realise a *high-dose drug* formulation, this effort is particularly important. Therefore, hollow microparticles show low density value (*e.g.*, spray drying feeds with high alcohol content) due to the formation of an empty structure.

Instead, particles with corrugated and wrinkled surface contribute to decrease the density of the powder, because this contributes to reduce the volume on the

$$Pe = \frac{\kappa}{8D_s}$$

Equation 7

outside (De Boer A. et al., 2017). As reported, spray-dried microparticles may show corrugated surface if the diffusion of the solvent within the spray droplet, regulated by the diffusion coefficient D_s of the dissolved substance, is higher than the drying rate κ constant, expressed in $cm^2 \times s^{-1}$ (Equation 7).

This behaviour could be obtained whether the feed solution to be spray-dried produces a single chemical phase (a solution) and therefore a better solubilisation of the drug, with a subsequent reduction of the Peclet number of the solutes. This allows estimating the Peclet number value (*Pe*): corrugated microparticles will be obtained when the *Pe* value is less than 1 (Figure 17).

In conditions in which drying rate κ (Equation 7) prevails, the solvent evaporates faster from the spray drops during the drying process, permitting the surface of the drop to enrich with the component associated with the highest Peclet number. Instead, a shell may be formed faster, due to the enrichment of the solid at the

$Pe \ll 1$ $Pe \gg 1$

Figure 17. Proposed particle formation during the spray drying process: for a low Peclet number, corrugated micropartcles will be formed; for a high Peclet number, a shell with a spherical shape will be formed.

surface of the drops, allowing for a more spherical shape of the final microparticles (Belotti S. et al., 2015; Vehring R, 2008).

METHODOLOGIES,

RESULTS AND DISCUSSIONS

Chapter 3

Part 1: RHEOLOGICAL PROPERTIES AND DRUG PERMEATION STUDY THROUGH CYSTIC FIBROSIS BRONCHIAL SECRETIONS

Based on the article:

Stigliani, M., **Manniello, M.D.**, Zegarra-Moran, O., Galietta, L., Minicucci, L., Casciaro, R., Garofalo, E., Incarnato, L., Aquino, R.P., Del Gaudio, P., Russo, P. "Rheological Properties of Cystic Fibrosis Bronchial Secretion and in Vitro Drug Permeation Study: The Effect of Sodium Bicarbonate". *Journal of Aerosol Medicines and Pulmonary Drug Delivery* 29, (2016).

3.1 Backgrounds and research aim

The present research was realised in collaboration with the *U.O.C. Genetica Medica* and *Centro Fibrosi Cistica* of *"Giannina Gaslini" Hospital* (Genoa). Several studies were conducted with the aim to evaluate the rheological properties of the CF sputum samples, and to improve the knowledge about the role of bicarbonate in the expansion process of mucins in the CF bronchial secretions. Finally, to understand the mechanisms that occur when inhaled micro- and nano-particles are deposited on the mucus and to check the ability of the drug to penetrate

Table 4. Parameters evaluated during the rheological studies.

$\eta = \frac{\tau}{\gamma}$	Viscosity (Pa s).	Viscosity η is defined as shear stress τ divided by shear rate γ.
G'	Storage modulus (Pa).	Represents the elastic portion of the viscoelastic behaviour, which describes the solid-state behaviour of the sample.
G"	Loss modulus (Pa).	Characterises the viscous portion of the viscoelastic behaviour, which represents the liquid-state behaviour of the sample.
$\tan\delta = \frac{G''}{G'}$	Loss factor or damping factor (dimensionless).	Describes the ratio of the two portions of the viscoelastic behaviour.

Adapted from: Mezger, T. G. *Applied rheology: With Joe Flow on Rheology Road*. (Anton Paar, 2015).

through CF mucus, we evaluated the dissolution and permeation properties through CF sputum of ketoprofen lysinate (Klys) a nonsteroidal anti-inflammatory drug, from a previously developed Dry Powder Inhaler (DPI).

Using a Two-Plate Model rotational rheometer, and the gel fractions of CF sputum, three parameters were investigated (Table 4): the complex viscosity, he *storage modulus* G', and the *loss modulus* G" (Mezger T., 2015).

3.2 Materials and methods

3.2.1 Materials.

Ketoprofen lysine salt was kindly donated by Dompe' spa (L'Aquila, Italy). Water was purified by reverse osmosis (Milli-Q, Millipore, France). L-Lysine and 2-Propanol (2-PrOH) USP grade was purchased from Sigma-Aldrich (Milan, Italy).

3.2.2 Rheological studies.

Rheological measurements were performed using an ARES rotational rheometer (Rheometrics, Inc.) with parallel plates geometry (plate diameter 25 mm, gap of 0.25 mm). Dynamic frequency sweep tests were conducted in the frequency range of 0.1–10 rad/s using a strain amplitude of 0.4%, proven to be in the linear viscoelasticity range (LVR) by means of strain sweep preliminary measurements (Shuster B. et al., 2014; Tang A. et al., 2012). Four parameters were identified dependent on frequency ω, rad/s: η^* (complex viscosity), G' (elastic modulus), G'' (viscous modulus), and tan d (ratio of G'' to G'). All samples were tested at 37 °C and all experiments were carried out under air flux.

For rheological tests, we followed the procedures to avoid saliva contamination (Puchelle E. et al., 1984; Rubin B.K., 2009) and only the gel fraction of the CF sputum was loaded onto the plate. The gel phase was separated from the small volume of liquid phase (saliva contamination) either by means of centrifugation (10 min at 11000 g) or by simply lifting it with a spatula, allowing the liquid phase to

drip off: no difference in rheological behaviour of the gel phase, depending on sputum separation method, was evidenced (data not shown).

Rheological studies were repeated on 1ml of untreated sputum (W, added with 100 μl of water) and on 1ml of sputum, from the same patient, treated in vitro with 100 μl of bicarbonate at a final concentration in the sputum ≈100 mM. No effect was observed using bicarbonate solutions with concentrations lower than 100 mM. Therefore, we decided to perform this study with 100 mM as the lower effective concentration. Samples were introduced in an incubator shaker at 37 °C for 30 min, in order to allow the mixing of water or bicarbonate with the sputum.

3.2.3 Ketoprofen Lysine spray-dried powders preparation.

Micronized particles, containing ketoprofen in its lysine salt form (Klys), were prepared by spray drying the drug, with leucine (85:15 w/w, respectively) as dispersibility enhancer from water/isopropyl alcohol (7:3) mixtures.

The drug and leucine were solubilized in water; then the organic solvent was added under continuous magnetic stirring. The total powder concentration reached 5% w/v. The resulting feed solution was dried using a Büchi mini spray dryer B-191 (Büchi Laboratoriums-Tecnik, Flawil, Switzerland) under the following operative conditions: inlet temperature 110 °C, outlet temperature ranged from 72 to 75 °C, drying air flow 500 L/min, aspiration rate 100%, air pressure 6 atm, feed rate 5 ml/min, nozzle 0.5 mm.

Spray-dried powders were collected and stored under vacuum for 48 h at room temperature. Production yield was expressed as weight percentage of the final product over the total amount of sprayed material.

Spontaneously produced sputum was collected from patients at the Cystic Fibrosis Center from the Istituto Giannina Gaslini (Genoa). Patients were in stable conditions, some of whom were receiving antibiotic treatment (tobramycin, ceftazidime, amikacin, and/or vancomycin). None of the patients received treatment with hypertonic solution or DNAse 4h before sputum collection.

3.2.4 Ketoprofen lysinate quantification.

Klys was quantified by UV detection (Evolution 201, ThermoFisher Scientific, Spectral, Ozzano dell'Emilia, Bologna, Italy) at a wavelength of 259 nm, using 1 cm SUPRASIL® quartz cell (Hellma 100-QS, HELLMA Italia srl, Milan). The analytic method was validated using standard solutions of Klys in the range of 5–30 μg/mL ($y = 0.0407x + 0.0048$; $R^2 = 0.9998$).

3.2.4 In vitro permeation study of a drug through CF sputum samples.

Permeation assays were performed by means of Franz-type vertical diffusion cells (Hanson Research Corporation, A, USA).

The cell system temperature was kept constant at 37 °C throughout the experiment by recirculating water from a thermostatically controlled bath. Continuous stirring at 170 rpm was provided by Teflon-coated stirring bars placed in the receptor compartment.

A thin layer (3 mm) of CF sputum was interposed between the synthetic membrane and the drug formulation (about 35 mg of powder exactly weighed). Samples (200 μl) were removed from the outer sampling port at defined time intervals (30, 45,

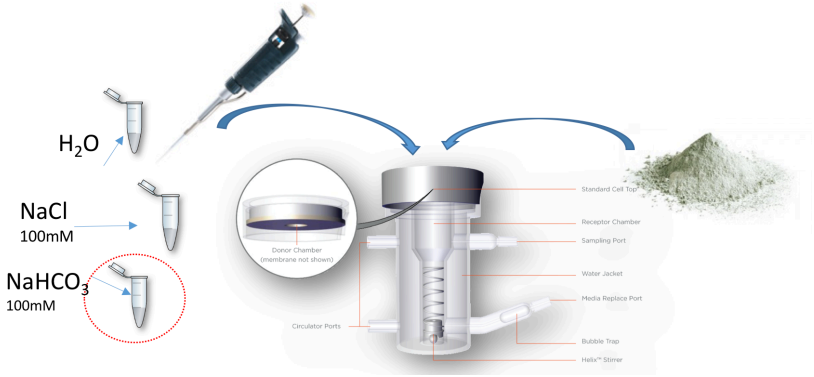

Figure 18. Schematic representation of the dissolution process of KLys after the pre-treatment of the CF sputum samples and the addiction of the powders, respectively, to the donor compartment of a vertical diffusion cell (Franz cell apparatus).

60, 90, 120, 150, and 180 min) and analysed (Figure 18).

The experiments were first conducted first on untreated sputum in order to see if and to what extent the sputum slows down the process of dissolution and permeation of the drug. Then, experiments were repeated on sputum after addition of 50 μl of a sodium bicarbonate solution to about 500 mg of CF sputum (final bicarbonate concentration in the sputum: 100 mM) and of 50 μl of an isosmotic NaCl solution, in order to obtain information about the effect of sodium bicarbonate on drug dissolution.

Permeation data were reported as the quantity of permeated drug per permeation area (mg/cm^2) related to time. The amount of the drug permeated per area (Q) for each time interval was calculated by means of the following equation (Equation 9):

$$Q\left(\frac{mg}{cm^2}\right) = \frac{V_R \times C_n + \sum_{i=0}^{n-1} V_p \times C_i}{A}$$

Equation 9

where V_R is the receiver volume; C_n is the drug concentration in the receiver at the time n; V_p is the volume of the removed sample; C_i is the drug concentration in the receiver at the time n-1; A is the permeation area (cm^2) (Russo P. et al., 2013).

3.3 Results and discussion

3.3.1 Rheological studies: effect of sodium bicarbonate.

In both normal and pathological conditions, the bronchial secretions are characterised by a non-Newtonian fluid behaviour, with a viscosity that decreases with the shear rate (Lafforgue O. et al., 2017).

For CF sputum, oscillatory rheological test was performed to mimic the *in vivo* stress conditions in the airways, minimizing the structural changes of the glycoprotein.

After the method setting-up, measurements on three sputum specimens, derived from three different CF patients, were conducted. As shown in Figure 19, the frequency-dependence of rheological parameters for the three sample was similar. The elastic modulus (G') was always greater than the viscous one (G'') (Figure 19a). Moreover, G' and G'' increased with frequency. Tan δ values (Figure 19c)

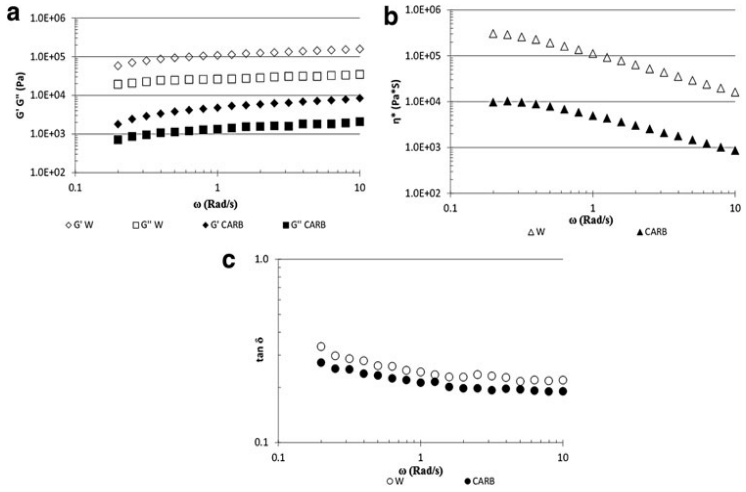

Figure 19. Rheological parameters of sputum samples treated in vitro either with bicarbonate (filled symbols) or with water (control, empty symbols) versus oscillation frequency (ω): (a) elastic and viscous moduli; (b) complex viscosity; (c) Tan δ. The curves shown are representative of three experiments done in similar conditions.

are sensibly less than 1, indicating an elastic behaviour higher than a viscous one (G'>G''). Considering the elastic characteristic of CF mucus and the force applied to the material, this test could be qualitatively compared to the stress applied to the mucus in CF airways by cilia beats.

On the basis of the previous considerations (§ 1.2.2), regarding the role of sodium bicarbonate in the physiological expansion of mucin, rheological studies were repeated using both 1ml of untreated sputum (W, added with 100 μl of water) and 1ml of sputum obtained from the same patient after the same specimen collection and treated *in vitro* with 100 μl of bicarbonate (CARB, final concentration in the sputum ≈ 100mM). The ratio of G'' to G' remains unchanged after the bicarbonate addition, as indicated by tan δ profiles almost overlapped (Figure 19c).

The complex viscosity decreased with increasing frequency, as for pseudo-plastic fluids, where the macromolecular components of the material align themselves in the direction of the applied force, reducing their resistance to the flow. The results were very encouraging, highlighting that the addition of sodium bicarbonate to the sputum sample caused a reduction in complex viscosity (Figure 19b) with a downward shift of both the elastic (Figure 18a, rhombi) and viscous moduli (Figure 19a, squares).

Because of the small amount of the biological material, the shown curves are representative of three experiments done in similar conditions.

3.3.2 In vitro permeation study of Ketoprofen lysinate (Klys).

Apart from deposition, the efficacy of an inhalation product depends also on its ability to interact and solubilise with the fluids lining the lung. Consequently, during

the development of a new dry powder inhaler (DPI), the *in vitro* dissolution profile is as well an essential way of anticipating the *in vivo* behaviour of powders, after the impaction on the respiratory tract. The dissolution study becomes even more significant in the case of diseases such as CF, characterized by the presence of a

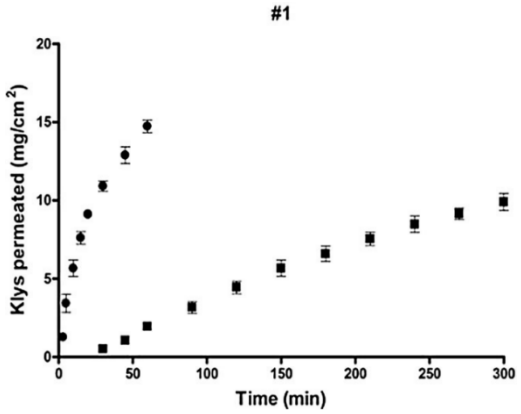

Figure 20. Sputum permeation profiles of ketoprofen lysinate (squares) in comparison with drug permeation profile without mucus (circles) (vertical bars are reported because the experiment has been done in duplicate, thanks to a larger amount of sample available).

particularly sticky pulmonary mucus (Albers G. et al., 1996).

To verify if the obtained reduction of the complex viscosity was related to a permeation enhancement of a drug through the CF sputum in the same experimental conditions, we investigated about the dissolution process of Klys DPI, developed in a previous work, using a vertical diffusion cell (Franz cell apparatus). Permeation experiments were conducted on seven sputum specimens (#1–#7), pouring the sputum onto the nitrocellulose membrane and the drug formulation on top of the sample.

Figure 20 shows the effect of CF sputum on drug dissolution and permeation of Klys interposing a thin layer of CF sputum between the nitrocellulose membrane and the drug formulation. The Franz vertical diffusion cells were used in their modified configuration. As expected, the sputum layer slowed down drug dissolution and permeation, acting as a physical barrier (Figure 20).

Afterwards, in order to evaluate the effect of bicarbonate on the sputum permeation of Klys from the DPI, the permeation experiments were repeated on the same seven specimens (#1–#7) but adding this time 50 μl of sodium bicarbonate solution on the sputum layer.

pH values of sputa changed, moving from 7.14 ±0.44 before to 7.61 ±0.40 after bicarbonate addition. All samples, except #4, showed that bicarbonate treatment leads to higher permeation of Klys across CF sputum in comparison to the untreated control, confirming the effect of bicarbonate in the reduction of mucus viscosity.

Finally, to be sure that bicarbonate action on sputum was not due to an osmotic effect, we analysed the permeation effect of 50 μl NaCl, isosmotic with $NaHCO_3$, on three sputum samples (#5–#7). Even if the viscoelastic properties of CF sputum were highly variable, leading to different transport capacity in 5 h (4–10 mg/cm^2), the results shown in Figure 21 indicate that the permeation profiles obtained with the sputum treated with NaCl solution and the untreated one are completely overlapping, confirming that bicarbonate effect was not related to a change in osmotic pressure.

Differently from bicarbonate, the addition of water or NaCl solution did not change sputum pH (from 7.24 ±0.38 to 7.24 ±0.43 after the addition of NaCl solution and from 7.08 ±0.30 to 7.08 ±0.30 after water addition).

Vertical bars are the graphical standard deviation of each measurement. It was possible to repeat the experiment when a larger amount of CF sputum sample from

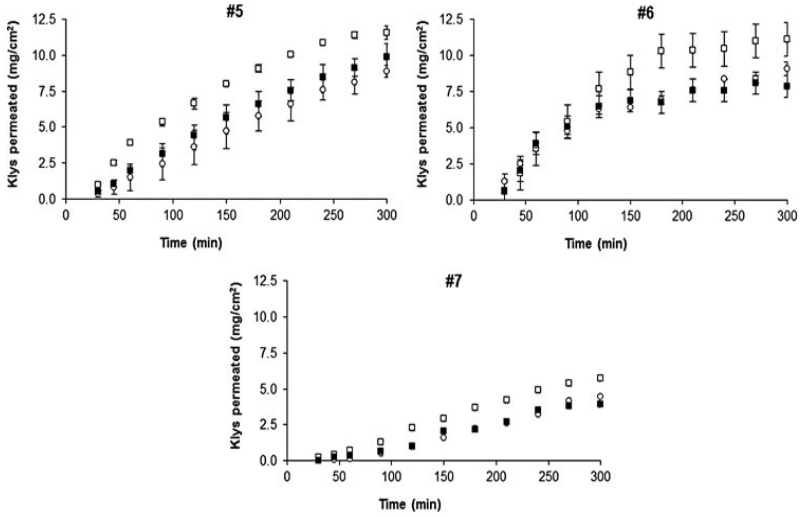

Figure 21. Permeation profiles of Klys through untreated (filled squares) and treated (empty squares) sputum from four different patient samples (vertical bars are standard deviations when the measurement has been done in duplicate or triplicate, because of a larger amount of sample available).

each patient was available. Thus, the measurement was done in duplicate or triplicate.

3.4 Conclusions

The thick mucus characteristic of CF, besides impairing the mucociliary transport and creating a favourable environment for bacterial infections, constitutes an important barrier to the dissolution of inhaled drugs.

The permeation studies showed that the CF sputum, a fluid with a pseudo-plastic behaviour characterized by a more elastic than viscous component, acts as an important barrier to drug dissolution/permeation, even in the case of Klys, an active compound very soluble in water.

In addition, both rheological and permeation studies offer another improvement in the knowledge about the role of sodium bicarbonate in mucin expansion process. This weak base appears to act by decreasing the high viscosity of CF bronchial secretions and, potentially, resulting in a better mucus clearance.

Chapter 4

Part 2 A: DRY POWDER FORMULATION: CLARITHROMYCIN VERSUS ITS HYDROCHLORIDE SALT

Based on the article:

Manniello, M. D., Del Gaudio, P., Porta, A., Aquino, R. P. & Russo, P. "Aerodynamic properties, solubility and *in vitro* antibacterial efficacy of dry powders prepared by spray drying: Clarithromycin versus its hydrochloride salt". *European Journal of Pharmaceutics and Biopharmaceutics*. 104, (2016).

4.1 Scientific background and research aim

Inhaled medications for a local action become important during the symptomatic treatment of chronic lung infections, which are the basis of the clinical issues affecting CF patients (Laube B. et al., 2014). In particular, on the surface of the CF lower respiratory tract an altered osmotic movement of water occurs leading to the production of thick and sticky secretions (Cuthbert A., 2011; Hoegger M. et al., 2014; Laube B. et al., 2014). These conditions induce the basis of the bacterial colonisation of the airways since in childhood, in particular due to *Staphylococcus aureus* and *Pseudomonas aeruginosa* species. Particularly in chronic infections, a typical secreted biofilm protects bacteria against the antibiotic action and the immune system of the host, causing also a persistent inflammatory state (Moss R., 2009; Nixon G. et al., 2002; Bjarnsholt T. et al., 2013 [1,2]).

Goals of the therapy against CF are to delay pulmonary disease progression and to maintain and restore respiratory function. Some of the current drug therapies include the use of various classes of antibiotics, administered via inhalation, alone or in combination (McCoy K. et al., 2008). Compared to liquid formulations, DPIs have some key advantages: they are easy to handle and to use. The medicine is administered as a micronized dry powder, more stable than liquids, in which hydrolysis reactions of functional groups such as esters, amides, lactones or lactams may occur (Ohtake S. and Shalaev E., 2013). In addition, the emission of the dose could be activated by patient's inhalation, and surely no propellants and solvents are required (§ 2.3).

Due to the overuse of the first-line antibiotic therapies, and considering a recognised lack of availability of new scaffolds (Payne D. et al., 2007; Tommasi R.

et al., 2015) (although new antibiotics are in human clinical trials, and a new inhaled dosage form proposal is under development by Polyphor and the European Innovative Medicines Initiative, specifically for CF; Bush K. and Page M., 2017; Moir D. et al., 2012; Nicolaou K. and Rigol S., 2017; Wright G., 2017), the elaboration of an unconventional pharmacological management, using long-standing molecules, could represent an alternative proposal and a helpful perspective to counteract the progression of the CF-related pulmonary worsening (Brown E. and Wright G., 2016; Dubourg G. et al., 2017; Falagas M. et al., 2016; Martens E. and Demain A., 2017; Rolain JM et al., 2016).

Following this need, the macrolides have gained a possibility to be considered in CF lung disease. Furthermore, they could cooperate with other bactericidal antibiotics administered routinely against *P. aeruginosa* (Fitzgerald N. and Jaffe A., 2016). It is well-known that the oldest semi-synthetic macrolide compounds, with a 14-, 15-, and 16- membered macrocyclic lactone (Fernandes P. et al., 2016), show antibiotic activity. Moreover, an assortment of immunomodulatory effects (Shinkai M. et al., 2005), together with the ability to attenuate at sub-inhibitory concentrations the quorum sensing (cell-to-cell signalling), as well as to interfere with the virulence factors expression (Imperi F. et al., 2009; Visaggio D. et al., 2015), contributed for years to a proper credit towards this class of antibiotics (Alhajlan M. et al., 2013; Majik M. and Parvatkar P., 2014; Saadat A. et al., 2014; Tan et al., 2016). This evidence has therefore guided physicians to trial the azithromycin (15-membered ring macrolide) in the clinical practice of the CF respiratory disease (Bell S. et al., 2005; Imperi F. et al., 2014; McArdle J. and Talwalkar J., 2007; Mogayzel Jr et al., 2013).

Given this scientific background, the purpose of the research herein presented was to obtain micronized powders of clarithromycin (CLM) to be administered to CF patients via inhalation. In order to exert its therapeutic action, a drug formulated as a dry powder for inhalation must accumulate in the site of action, and dissolve into the fluids lining the lung. To fulfil bot conditions, is necessary an accurate tuning of the powder aerodynamic diameter and of the solubility of the active compound. However, CLM is characterized by a very poor water solubility which can impair the drug local activity (experimentally evaluated, 0.6 mg/ml at 37°C, after 72 h in a phosphate buffer 50 mM with few drops of KOH 1 M to reach the value of 6.75. Manniello M.D. et al., 2016). One of the common strategies for increasing drug solubility could be its micronisation and amorphisation by spray drying (Yonemochi E. et al., 1999), a one-step process able to produce dry powders from solutions or suspensions, with a good control over particle size, morphology and powder density. These factors are well known to influence the aerosolisation properties of dry powders. Indeed, spray drying has been used by a number of researchers to generate dry powders suitable for inhalation (Adi H. et al., 2010; Geller D. et al., 2011; Jensen D. et al., 2010; Leung S. et al., 2016; Zhou Q. et al., 2014). Moreover, the dimethyl-amino group on CLM structure can be salified for solubility improvement (pKa = 8.99) (“Clarithromycin,” 2008). Therefore, we produced DPIs containing CLM via spray drying by a fine setting of the process parameters and with the dual purpose of improving both the aerodynamic properties and the solubility of the commercially available drug.

4.2 Materials and methods

4.2.1 Materials.

Clarithromycin raw material (CLM) was purchased from Carbosynth Limited (Berkshire, UK).

Water was purified by reverse osmosis (Milli-Q, Millipore, France). 2-Propanol (2-PrOH) USP grade was purchased from Sigma-Aldrich and hydrochloric acid 37% was purchased from Carlo Erba Reagents (Milan, Italy).

Size 3 gelatin capsules were purchased from Farmalabor (Canosa di Puglia, Italy).

The device used for aerodynamic tests was the monodose DPI RS01 model 7 with a low resistance to airflow, kindly donated by Plastiape® SpA (Lecco, Italy).

4.2.2 Preparation of CLAHCl and CLA batches (A)

Clarithromycin micronized powders (CLA) were prepared from different hydro-alcoholic solutions (containing 2-Propanol from 30 to 70% v/v), with a total powder concentration ranging from 1% to 5%. CLA batches were prepared by adding CLM to the different hydro-alcoholic solutions under continuous magnetic stirring.

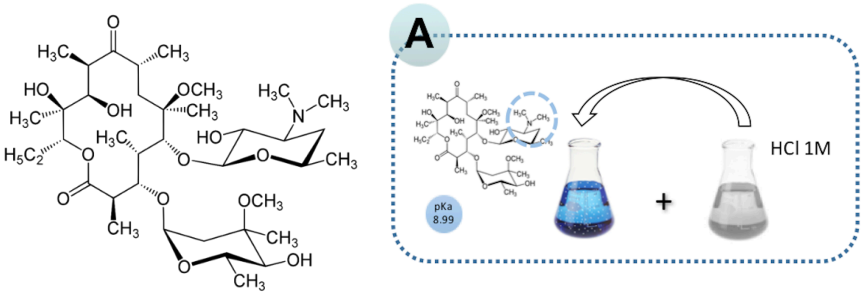

Figure 22. Schematic representation of the preparation method for the CLAHCl batches.

CLAHCl batches containing the antibiotic in its hydrochloric salt form were obtained neutralizing the liquid feed with few drops of hydrochloric acid (HCl) 1 M, until reaching a pH of 6.5 ±0.1 (Figure 22).

All liquid feeds were dried using a Büchi Mini Spray Dryer B-191 (Büchi Laboratoriums Tecnik, Flawil, Switzerland) under the following operative conditions: inlet temperature 90 °C or 110 °C, according to alcohol content, outlet temperatures from 65 °C to 75 °C, drying air flow 500 L/min, aspiration rate 100%, air pressure 6 atm, feed rate 5 ml/min, nozzle 0.5 mm. Each preparation was carried out in triplicate. All the SD powders were collected and stored at room temperature. Production yields were expressed as percentage of the final product compared to total amount of the material sprayed. Powders produced were solubilized in CH_3CN/H_2O (1:1 v/v) and analysed in terms of drug content by means of HPLC method described below.

4.2.3 Clarithromycin quantification.

CLM determination by HPLC followed an adapted United States Pharmacopoeia method. Briefly, 15 mg of CLM raw material was solubilized in 100 ml of CH_3CN/H_2O (1:1) until complete dissolution. The solution was analysed by HPLC at a wavelength of 205 nm (Chromatopac L-10AD system equipped with a Model SPD-10AV UV–vis detector and a Rheodyne Model 7725 injector loop 100 μl, Shimadzu, Kyoto, Japan). The analytical mobile phase consisted of CH_3CN and phosphate buffer 50 mM (2:3) adjusted to pH 6.8 ±0.1 with potassium hydroxide 1 M. The saline buffer was then filtered with 0.45 μm filters. Peak areas were calculated with a Shimadzu C-R6A integrator. A calibration curve was worked out

and the linearity between CLM and AUC was calculated in the range of 40 – 300 μg/ml ($y = 9.708x + 1.2647$; $R^2 = 1$).

4.2.4 Particle size.

Particle size of both raw materials (CLM) and engineered particles (CLA, CLAHCl and CLANAC batches) was determined using a light scattering laser granulometer, equipped with a Tornado powder dispersing system (LS 13320 Beckman Coulter Inc., FL, USA). The LS 13320 uses a 5 mW laser diode with a wavelength of 750 nm and reverse Fourier optics incorporated in a fibre optic spatial filter and binocular lens systems. Particle size distributions were calculated by the instrument software using a Fraunhofer model. Powder samples were measured using a dry powder dispersing unit, without using any solvent which can alter powder surface properties. Samples were charged into a plastic cylinder in order to obtain an obscuration value between 4% and 8%. Results were expressed as d_{50} and Span. The Span value was calculated as follows (Equation 10):

$$Span = \frac{d_{90} - d_{10}}{d_{50}}$$

Equation 10

4.2.5 Microparticles morphology.

Morphology of CLM, CLA base, CLAHCl and CLANAC microparticles was examined using a scanning electron microscope (SEM) Zeiss EVO MA10 with a secondary electron detector (Carl Zeiss SMT AG, München-Hallbergmoos,

Germany), operating at 14 kV, equipped with a Leica EMSCD005 metallisator and producing a deposition of a 200–440 Å thick gold layer (Del Gaudio P. et al., 2014).

4.2.6 Aerodynamic behaviour of CLAHCl, CLANAC and CLA spray-dried powders.

The *in vitro* aerodynamic properties of the spray-dried powders were evaluated as reported in the European Pharmacopoeia 8th Ed., following the method *"Aerodynamic assessment of fine particles in preparations for inhalation".*

The *in vitro* aerodynamic properties of the spray-dried powders were carried out using a single-stage glass impinger (Glass Impinger, SSGI; Apparatus A, Eur. Pharm. 8th (20918) Copley Scientific Ltd., Nottingham, UK. Figure 23) and the monodose DPI RS01 model 7, kindly donated by Plastiape®, as device for aerosolise the spray-dried powders. The device is a breath activated, reusable

Figure 23. Glass Impinger; Apparatus A Eur. Pharm. 8th (20918). Copley Scientific Ltd., Nottingham, UK.

DPI, working with a single unit size 3 capsule. The capsule is horizontally inserted into the pulverization chamber and pierced by two needles at the bottom and at

upper side: the inhaled air creates a turbulence that shakes and twists the capsule, facilitating its emptying.

For the SSGI experiments, 30 ml and 7 ml of a suitable solvent were introduced in the lower and upper stages of the Glass Impinger, respectively, in order to solubilize and quantitatively recover the deposited drug in both form, micronised (CLA) or salified batches. Hard gelatine, clear and colourless capsules size 3 were filled manually with 30.0 ±0.5 mg of spray dried powder. Then, the capsule was introduced into the device and pierced twice. The vacuum pump was operated at a flow rate of 60 ±5 L/min for 5s (Erweka vacuum pump VP 1000 equipped with an electronic digital flowmeter type DFM, Erweka Italia, Seveso, MI, Italy).

To recuperate the amounts of the CLAHCl and CLANAC deposited powder, each impingement chamber of the SSGI was washed with purified water (Milli-Q, Millipore®), while the solvent used to recuperate CLA batch was CH_3CN/H_2O (1:1). CLM and NAC quantifications were performed by HPLC-DAD method, as described. To achieve samples dilutions, a CH_3CN/H_2O solution (1:1) was used.

Each deposition experiment was performed on 10 capsules (Eur. Pharm. 8^{th}, 20918) and repeated in triplicate. Upper and lower parts were washed with CH_3CN/H_2O (1:1) in order to recover the powder deposited on each part; the drug quantification was performed by HPLC as described.

The Delivered Dose (DD), which provides information about the cohesiveness of the powder, was gravimetrically determined and expressed as percentage of powder exiting the device *vs.* amount of powder introduced into the capsule.

The Fine Particle Fraction (FPF, called often *respirable fraction*), is expressed as a percentage and is defined as ratio of the drug characterized by particles with an aerodynamic diameter smaller than 6.4 μm which passed into the lower

impingement chamber *vs.* total drug amount charged into the capsules. The FPF epitomises the fraction of the drug that is available to exert its pharmacological action in the target site, that in this study is represented by the conducting zone of the lung. From a formulative point of view, the greatest efforts are focused on the ability of the powder to generate a high FPF, which represent a starting point to evaluate the aerodynamic behaviour among different batches. Moreover, FPF values higher than 30% represent a good aerosol performance index.

4.2.7 Solubility assessment of CLM in CLAHCl and CLA batches.

Solubility of the drug CLM, in CLM (raw material), CLA and CLAHCl powders was evaluated according to USP method in phosphate buffer 50 mM (pH 6.75 ±0.05; *buffer A*).

An excessive amount of powder was introduced into glass vials containing 5 ml of buffer A; the samples were stirred and stored at 37 °C for 72 h. After that, samples supernatants were filtered with 0.45 μm filters and the concentration of dissolved drug was determined by HPLC method as described before. The solubility measurements were performed in triplicate.

4.2.8 Dissolution profile of clarithromycin.

The *in vitro* dissolution study of the spray-dried microparticles was performed by a Vertical Diffusion Cell system (VDC; Hanson Research Corporation, CA, USA. Figure 24), as reported elsewhere (Russo P. et al., 2013). The receptor chamber (RC; standard volume – 7 ml) was filled with buffer A, to create a positive meniscus. Between the donor and the receiving chamber, a filter of nitrocellulose (MFTM

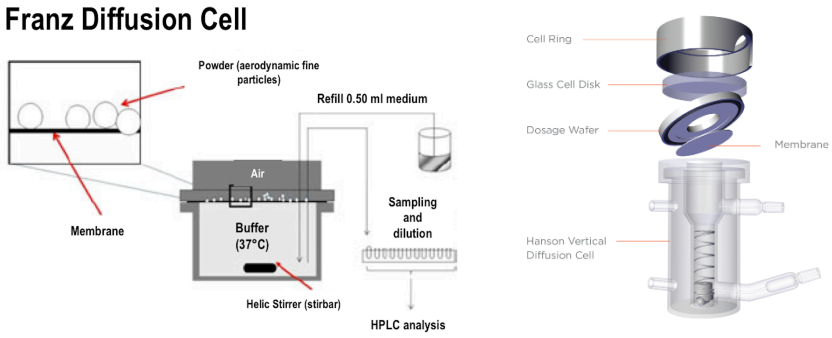

Figure 24. Schematic representation of the vertical diffusion cell method used to evaluate the dissolution process of CLM within the powders (left). The Franz cell apparatus used to perform the experiments. Adapted from Hanson Vertical Diffusion Cell system (right).

Membrane filters; filter type 0.45 μm HA), previously wetted with few drops of buffer A was interposed; on the top of the membrane a single dosage wafer was placed. Having ensured the sealing of the Donor Chamber (DC) by the glass cell disk and the cell ring, the VDC system was let warming at 37 °C maintaining the medium under continuous magnetic stirring (170 rpm) in the RC. After 30 min, to ensure the sink conditions, about 5 mg of both CLA and CLM powders accurately weighted and were transferred directly in the DC; the DC was then sealed and finally the dissolution experiment started. At pre-determined time points, 500 μl of the dissolution medium was sampled by adding fresh buffer A, constantly warmed throughout the experiment. Quantitative analyses of the dissolution media were conducted in triplicate using the previously described HPLC-DAD method, and reporting the dissolution data as the percentage of permeated drug related to time.

4.3 Results and discussion

4.3.1 Manufacturing and characterization of spray-dried powders.

It is well known that the addition of an organic solvent into the liquid feed lowers the surface tension of the droplet during the atomization process, with a significant change in Peclet number (Pe) of the solute (Vehring R., 2008). In fact, the diffusion of the solvent mixture through the droplet can become faster than the particle formation in the drying chamber. This consequence strongly affects both particle size and morphology of the spray-dried microparticles, that influence the aerosolisation properties of the dry powders. In our study, the Pe was also modified by changing the pH of the liquid feed processed: the addition of HCl 1M, in fact, occur to both increase the solubility of the drug and to reduce the Pe of CLM.

With the aim to optimize the aerodynamic properties of clarithromycin dry powders, by modifying the drug solubility (acting on the pH of the liquid feed) and the surface tension of the droplet (acting on alcohol content), several spray-dried batches were produced: CLA batches containing the drug as a free base (micronized and spray-dried raw material, indicated as CLA), and CLAHCl batches containing the drug with the dimethyl-amino functional group (basic portion) in its protonated form.

In accordance with CLM hydrophobic nature, liquid feeds containing the non-salified drug had the appearance of suspensions, with a precipitate in a quantity dependent on CLM concentration.

Adding few ml of HCl 1 M to these suspensions, up to a pH value of 6.50 $\pm$0.05, clear solutions were obtained, demonstrating the drug salification. CLA batches were obtained from drug suspension with a good process yield (> 60%). CLAHCl

batches dried from drug solutions showed process yield generally higher than CLA batches indicating an improvement in powder flow properties (Table 5).

Table 5. Physical characteristics of co-spray-dried powders: feed solution compositions, yield of the process and particle size.

Batch #	Büchi Mini Spray Drier B-191					
	Spray Drying Feeds				Yield %	d_{50} μm (Span)
	Feed % (p/v)	2-PrOH / H_2O (ml)	n:n	pH		
CLA	3.0	50/50	-	10.50 (± 0.05)	57.0 ± 6.3	4.4 (2.2)
CLA HCl#1	2.0	70/30	-	6.50 (± 0.05)	70.1 ± 2.8	3.3 (1.9)
CLAHCl#2		60/40			72.5 ± 1.5	3.3 (2.0)
CLAHCl#3		50/50			70.5 ± 1.0	3.2 (2.0)
CLAHCl#4		40/60			73.1 ± 3.5	2.8 (2.3)
CLAHCl#5		30/70			71.5 ± 0.9	3.4 (2.1)

The particle size of the spray-dried fine particles was determined by a light-scattering laser granulometer equipped with a *Tornado Powder* Dispersing System unit.

As to powder dimension, the drug solubilisation in the liquid feeds led to a sensible reduction in particle size: in particular, CLAHCl #4 dried from hydro-alcoholic solution containing 40% v/v of isopropyl alcohol showed the smallest median

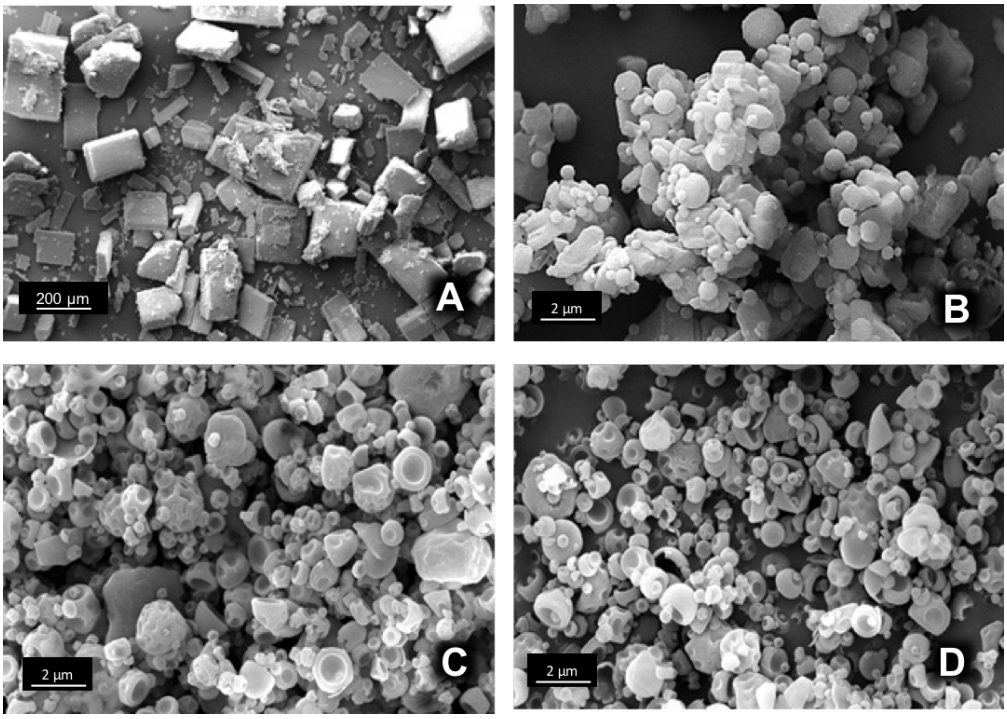

Figure 25. SEM pictures of Clarithromycin Raw Material (A); spray-dried particles obtained at pH 10.50 (B: CLA) and at pH 6.50 (C: CLAHCl #3; D: CLAHCl #4).

diameter (Table 5; d_{50} = 2.80 μm). The drug salification had important effect also on particle morphology, thanks to the reduction in the Pe: during the spray drying process, the CLM surface enrichment and droplet saturation are slower than solvent evaporation, with formation of wrinkled particles.

In fact, SEM micrographs of CLAHCl micronized particles showed highly wrinkled particles well separated and with no residual crystals (CLAHCl #3, CLAHCl #4 Figure 25 C and D). In contrast, SEM micrographs of CLA batches, showed almost spherical particles with continuous and uniform surface but bonded to larger and non-spherical particles, similar in shape to the raw material crystals (CLA #2 Figure 25 B).

Less influence of drug form in liquid feeds was evidenced for powder density values, all ranging between 1.23 g/cm^3 and 1.27 g/cm^3.

4.3.2 Aerodynamic behaviour of the CLM microparticles.

The *in vitro* deposition studies showed that drug content in salt form strongly influenced the aerodynamic performance of the spray-dried (SD) powders. High recovery percentages from the impaction apparatus were obtained (> 70%).

Table 6.

In vitro Deposition Study using a Glass Twin Impinger (Eur. Phar. 8th). Fine Particle Fraction (FPF) and Delivered Dose (DD) of CLAHCl spray-dried microparticles.

Batch #	FPF %	DD %
CLA	29.1 ± 1.0	99.9 ± 1.0
CLA HCl 1	50.5 ± 3.1	98.4 ± 1.6
CLA HCl 2	40.2 ± 4.7	96.8 ± 2.2
CLA HCl 3	41.0 ± 1.4	96.0 ± 1.3
CLA HCl 4	42.5 ± 1.7	91.8 ± 4.1
CLA HCl 5	40.0 ± 2.4	95.7 ± 1.2

Device used to aerosolize the powders: monodose (unit-dose pre-metered in capsule) DPI RS01 model 7, breath activated DPI; Plastiape®.

As shown in Table 6, the delivered dose (DD), defined as the dose delivered from the inhaler (Eur. Phar 8th 0671) and gravimetrically determined, was higher than 90% in all tested powders, a sign of a good powder dispersibility, with a low decrement for SD batches obtained from feed solutions with a higher water content. However, for CLA batches, this optimal DD values were not associated with equally good FPF values.

We attributed this powder behaviour to the higher diameter of CLA (Table 6): larger particles are also free flowing, but are not suitable for inhalation.

Differently, we observed a marked improvement of powder technological properties with CLAHCl batches. These batches containing smaller and wrinkled particles, conserved a good flowability and showed very high fine particle fractions. In particular, a higher content of 2-PrOH in processed liquid feeds led to the highest FPF values (CLAHCl #1).

4.3.3 Solubility assessment of CLM.

After deposition in the lower tract of the respiratory system, the drug has to dissolve in order to begin its action against susceptible bacteria, which are often organized

Table 7.
Exceeded Amount of each formulation in 5 ml of phosphate buffer 50 mM, 37 °C; pH 6.75.

Batch #	Solubility of CLM (mg/ml).
CLM	0.6 ± 0.02
CLA	0.7 ± 0.02
Practically insoluble.	
CLA HCl 1	10.1 ± 0.18
CLA HCl 2	6.1 ± 1.57
CLA HCl 3	11.4 ± 1.21
CLA HCl 4	9.9 ± 2.55
CLA HCl 5	11.8 ± 1.42

in biofilms and concentrated in fluids difficult to permeate, like CF mucus. Thus, a good water solubility of the SD powders represents a key factor to ensure the drug activity into the pulmonary secretions. Similar to CLM (raw material), powders obtained from alkaline feed suspensions (CLA) showed low solubility in a phosphate buffer 50 mM (Table 7; CLM, CLA).

A substantial increase in drug solubility (up to 11 mg/ml) was obtained with powders dried from feed solutions containing clarithromycin hydrochloride (Table 7, CLAHCl #1-#5).

4.3.4 Dissolution study.

To study and compare the behaviour of different SD powders when in contact with small amount of fluids (closer to *in vivo* conditions), we used a vertical diffusion cell

equipment (Franz cell apparatus), which allowed us to follow the drug release at different time points.

Official Pharmacopoeias, however, do not describe a standardised method to evaluate the dissolution process of a dry powder for inhalation. As reported by several authors, a vertical diffusion cell apparatus represents a proper tool to evaluate the drug dissolution, determining the amount of drug permeated through a synthetic membrane (May S. et al., 2014; 2012).

Dissolution studies were performed on batches #1 and #4, selected among powders prepared from hydro-alcoholic solutions containing 2% w/v of CLM, and

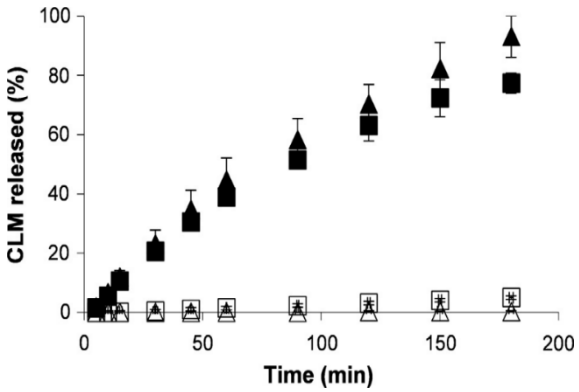

Figure 26. Dissolution profile of clarithromycin through nitrocellulose membrane from CLM raw material (empty triangles), CLA (empty squares), CLAHCl #1 (full triangles) and CLAHCl #4 (full squares). (n = 3).

also because of their higher FPF values.

Powders of raw material (CLM), CLA and the selected CLAHCl batches were tested, spreading 5.0 mg ±0.1 accurately weighted of each material in the donor compartment. The amount of powders was calculated in accordance of the solubility values, acquires experimentally, to ensure the sync conditions. In fact, to ensure this, the amount of dissolved drug does not have to influence the dissolution rate. So, the amount of powder to use in this experiment did not exceed the 10% of the solubility value.

As expected, CLA batches (drug in non-salt form, Figure 26, empty squares) showed dissolution profiles similar to the raw material (Figure 26, empty triangles), with a very low drug release during time. On the contrary, in accordance with solubility studies described above, higher dissolution profiles were obtained when CLAHCl batches were tested (Figure 26, full symbols).

These results confirmed that the spray drying process, together with the drug salification, enhanced both the CLM solubility and wettability of the powders, with no need of excipients, potentially toxic for a pulmonary drug delivery.

4.4 Conclusions

Thanks to a fine tuning of the spray dryer parameters and of the liquid feed composition, no excipient was necessary to obtain powders with a good yield (up to 73.0%), suitable aerodynamic properties (FPF up to 50.5%) and increased water solubility. The change in pH of hydro-alcoholic solution, from 10.5 to 6.5, was the breakthrough solution to achieve not only aerodynamic properties suitable for the powder deposition in the deep lung, but also a better dissolution of the drug into the liquid lining the lung.

Chapter 5

Part 2 B: CLARITHROMYCIN AND N-ACETYLCYSTEINE CO-SPRAY-DRIED POWDERS FOR PULMONARY DRUG DELIVERY: A FOCUS ON DRUG SOLUBILITY

Based on the article:

Manniello, M. D., Del Gaudio, P., Aquino, R. P. & Russo, P. "Clarithromycin and N-Acetylcysteine co-spray-dried powders for pulmonary drug delivery: A focus on drug solubility".
International Journal of Pharmaceutics (2017).

5.1 Scientific background and research aim of the CLANAC formulation

The most important mark that characterizes the CF disease is the production of very viscous secretions, which affect several organs. The related pulmonary disease develops consequently to the stasis of highly dense and sticky mucus, which disables over the years the mucociliary clearance. As a matter of fact, the chronic inflammation and persistent bacterial infections are related to the overexpression of the mucin proteins in the lower airways, that become highly glicosilated and denser during time (Ermund A. et al., 2015; Kreda S. et al., 2012; Robinson M. and Bye P., 2002; Rose M.and Voynow J., 2006; Yuan S. et al., 2015).

As reported, the drug micronisation and amorphisation obtained by spray drying technology is not able, alone, to enhance the *in vitro* solubility and dissolution rate of CLM. As showed above (Manniello M.D. et al., 2016), it has been demonstrated that, differently from the drug micronisation, drug salification for a pulmonary drug delivery was able to effectively improve the CLM solubility. Taking this into account, the aim of this research was to exploit the drug salification by means of a molecule having both an acidic moiety and a therapeutic benefit in the CF management. The N-Acetyl-L-cysteine (NAC), due to its well-known mucolytic activity and with a carboxylic functional group, was selected as salifying agent. The co-spray drying method was selected with the aim to obtain, in one step, a dry powder for inhalation characterised by a homogeneous distribution of the drugs in the microparticles and favourable aerodynamic properties.

5.2 Materials and methods

5.2.1 Materials.

Clarithromycin raw material (CLM) was purchased from Carbosynth Limited (Berkshire, UK) and N-Acetyl-L-cysteine was purchased from Sigma-Aldrich (Milan, Italy). Water was purified by reverse osmosis (Milli-Q, Millipore, France). 2-Propanol (2-PrOH) USP grade was purchased from Sigma-Aldrich.
Hypromellose (HPMC) capsules size 3 were kindly donated by Qualicaps® (Alcobendas, Spain). The device used for aerodynamic tests was the monodose DPI RS01 model 7 with a low resistance to airflow, kindly donated by Plastiape® SpA (Lecco, Italy).

5.2.2 Preparation of CLANAC batches (B).

CLM (MW = 747.95 g/mol) and NAC (MW = 163.20 g/mol) constituted the active ingredients of the micronized powders. The macrolide CLA has in its structure a basic amino-sugar, directly linked to its aglycone portion. This CLA basic moiety could be exploited to an ionization with NAC, characterized by an acidic moiety. Each single feed was obtained by gradually adding the NAC water solution to the CLM alcoholic suspension (2-Propanol), under continuous magnetic stirring (Figure 27). After pouring the NAC solution into the CLM suspension, clear water-alcoholic solutions, characterized by a pH of 6.50 ±0.5 were obtained and processed by spray drying technology.

Each batch was carried out in triplicate by spray-drying the hydro-alcoholic

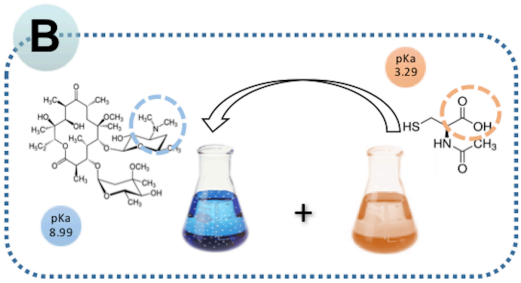

Figure 27. Schematic representation of the preparation method for CLANAC batches.

solutions containing 2-Propanol (from 20% to 50% v/v), using a Büchi Mini Spray Dryer B-191 (Büchi Laboratoriums Tecnik, Flawil, Switzerland) at the following operative conditions: the inlet temperature was fixed at 100 °C for CLANAC batches and at 90 °C for CLA batches, respectively. The drying air flow at 500 L/min, aspiration rate at 100%, air pressure at 6 atm, feed rate at 5 ml/min and nozzle diameter at 0.5 mm. The outlet temperature ranged between 65 °C and 68 °C for CLANAC batches, while 59 °C and 61 °C for CLA batches. Production yields were expressed as percentage of the final product compared to total amount of the sprayed material; all the spray-dried powders were collected and stored at room temperature (Aquino R. et al., 2012).

5.2.3 Clarithromycin and N-acetylcysteine quantifications.

CLM and NAC quali/quantitative determinations followed a method adapted from literature reports (Ourique A. et al., 2014; Shahbaziniaz M. et al., 2013). Briefly, CLM and NAC raw materials were solubilized separately in a CH_3CN/H_2O solution (1:1); then, the sample dilutions were analysed using the same HPLC-DAD method (Chromatopac L-10AD system equipped with a Rheodyne Model 7725 injector loop

100 μl, Shimadzu, Kyoto, Japan) in order to obtain two calibration curves. The analytical mobile phase consisted of CH_3CN and phosphate buffer 50 mM (37:63) adjusted to pH 4.50 ±0.05 with few drops of phosphoric acid 1%. The saline buffer was then filtered with 0.45 μm filters. Peak areas were calculated with a Shimadzu C-R6A integrator. A linear response over the concentration range of 40–300 μg/ml ($y = 9.4936x - 17.87$; $R^2 = 0.999$) was obtained for CLM and over the concentration range of 5–30 μg/ml ($y = 88.331x + 245.65$; $R^2 = 0.9929$) for NAC. The UV detection was performed at 205 nm for both analytes (Model SPD-10AV UV–vis detector).

5.2.4 Particle size of CLANAC batches.

Particle size of CLANAC batches was determined using a light scattering laser granulometer, equipped with a Tornado powder dispersing system (LS 13320 Beckman Coulter Inc., FL, USA), as reported above (§ 4.2.4).

Results were expressed as d_{50} and Span. The Span value was calculated as follows (Equation 10):

$$Span = \frac{d_{90} - d_{10}}{d_{50}}$$

Equation 10

5.2.5 Microparticles morphology.

Morphology of CLANAC microparticles was examined as reported above (§ 4.2.5), using a scanning electron microscope (SEM) Zeiss EVO MA10 with a secondary electron detector (Carl Zeiss SMT AG, München-Hallbergmoos, Germany), operating at 14 kV, equipped with a Leica EMSCD005 metallizator producing a deposition of a 200–440 Å thick gold layer.

5.2.6 FT-IR analysis of CLANAC microparticles.

Infrared analysis was performed using a FT-IR spectrophotometer (FT-IR/NIR Frontier Spectrophotometer, Perkin Elmer, MA, USA) equipped with a single reflection horizontal ATR accessory having a diamond coated Zn Se top-plate crystal fixed at incident angle of 90° (Universal ATR Accessory, Perkin Elmer, MA, USA).

CLANAC powder samples were analysed using 128 scan with a 1 cm^{-1} resolution step. ATR effect and atmospheric contributions from carbon dioxide and water vapour were corrected by the Spectrum1 software. Each analysis was carried out in triplicate, and results averaged.

5.2.7 Solubility of CLM in CLANAC batches

The addition of NAC in equimolar ratio into the feed solution led to a complete solubilisation of CLM as effect of an acid (NAC) base (CLM) interaction: when the water solution of NAC was gradually added to the alcoholic cloudy suspension of CLM, it became more and more clear. When all the NAC-solution was added, the final liquid feed was clear, thus, a solution was generated. In order to verify if the

obtained spray-dried powders increased their water solubility, compared to the unprocessed drug or to the CLA sprayed alone, a solubility study on the CLANAC co-spray dried powders, and as control on CLM raw material and on CLA spray-dried powder was performed. The solubility equilibrium in the experimental condition, was reached using an excess of powders and 5 ml of buffer A, composed as reported above (§ 4.2.7) and constantly warmed at 37 °C in a water bath; after 72 h samples were filtered and appropriately diluted with buffer A prior to be analysed. CLM quantification was realized by HPLC-DAD method, as described above (§ 4.3.3).

5.2.8 Dissolution profile of clarithromycin in CLANAC formulation.

The dissolution process of CLM in the CLANAC formulation was performed as reported (§ 4.2.8), but transferring about 6 mg of CLANAC powders in the DC to ensure the sink conditions of the experiment.

5.3 Results and discussion

5.3.1 Manufacturing and characterization of CLANAC spray-dried powders.

CLM (basic moiety) and NAC (acid moiety) were let able to interact, adding a water solution of NAC to an alcoholic suspension of CLM. In accordance with the stoichiometry of the reaction, the neutralisation occurs when the same moles number of CLM and NAC reacts. Thus, to achieve a final drug composition of about 3% (w/v) in each liquid feed, 0.545 g of NAC (3.34×10^{-3}) dissolved in purified

water and added to 2.50 g of CLM (3.34×10^{-3} moles) suspended in 2-PrOH. Each batch was produced by varying the solvent composition from 50% up to 80% of H_2O, in order to reduce the static electricity of the resulting powder and to ensure the solubilisation of the ionised drug.

As shown in Table 8, the process yield increased (up to 67.1%) with the 2-PrOH content in the spray drying feed solutions due to the easier evaporation process of the solvent during spray drying.

Particle size, on the contrary, decreased up to d_{50} = 2.60 μm by increasing the

Table 8. Physical characteristics of co-spray-dried powders: feed solution compositions, yield of the process and particle size.

Batch #	Büchi Mini Spray Drier B-191					
	Spray Drying Feeds				Yield %	d_{50} μm (Span)
	Feed % (p/v)	2-PrOH / H_2O (ml)	n:n	pH		
CLA	3.0	50/50	-	10.50 (± 0.05)	57.0 ± 6.3	4.4 (2.2)
CLANAC#1	3.0	50/50	1:1	6.50 (± 0.5)	67.1 ± 2.3	3.0 (2.2)
CLANAC#2		40/60			63.7 ± 5.9	2.7 (2.3)
CLANAC#3		30/70			57.7 ± 7.8	2.6 (2.1)
CLANAC#4		20/80			56.7 ± 1.1	3.3 (2.0)

The particle size of the spray-dried fine particles was determined by a light-scattering laser granulometer equipped with a *Tornado Powder* Dispersing System unit.

water content of the feed solutions (CLANAC #3; Table 8). The aqueous prevalence in the liquid feed probably contributed to a better solubilisation of the drugs (CLM and NAC), with a subsequent reduction of the *Pe* of the solutes, as reported elsewhere (Vehring R., 2008).

To complete the experimental evaluation, other two liquid feeds with 10% and 0% of 2-PrOH were prepared. In both cases, however, suspensions were obtained, too coarse to be processed by spray drying process. In addition to the volume diameter and the density, the shape of the microparticles for inhalation has an important role in their aerodynamic behaviour, as evidenced by the equation of the aerodynamic diameter (d_{AE}; Equation 1), where χ is the particle shape observed by means of SEM analysis.

In particular, particles smooth and spherical in shape have a χ factor next to 1, while corrugated particles showing many wrinkles have increased the χ shape factor value. The morphology studies showed an increase in particle corrugation as an effect of the NAC presence in the spray drying feed solutions, which is likely to improve the aerodynamic performance (Figure 28).

As well known, the morphology of the spray-dried particles is influenced by the

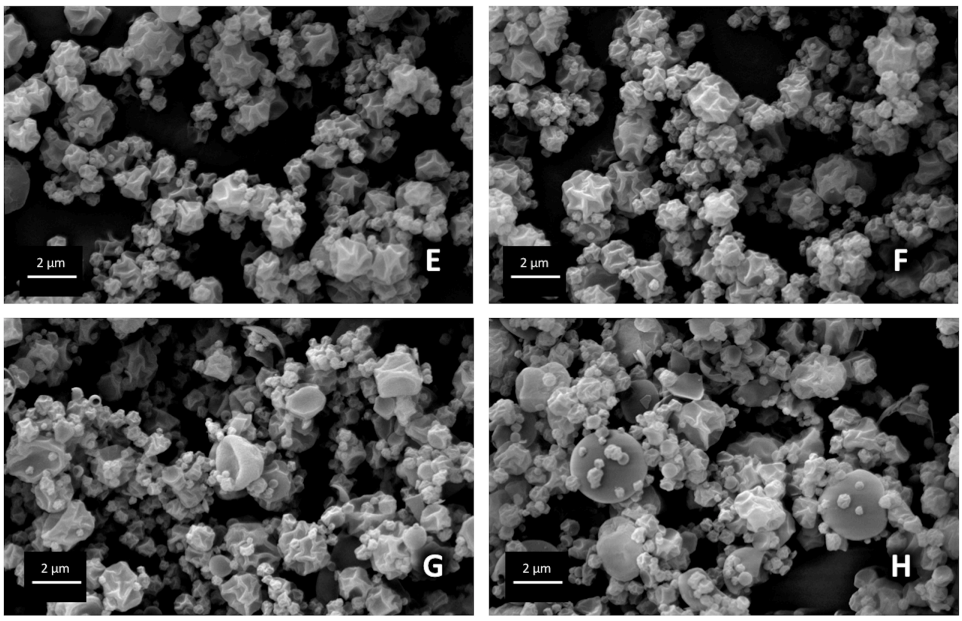

Figure 28. SEM micrographs of co-spray-dried CLANAC microparticles. Wrinkled micronized particles dried from 2-PrOH/water 20:80 (E) and 30:70 (F) (v/v). Blends of wrinkled and spherical particles dried from 2-PrOH/water 40:60 (G) and 50:50 (H) (v/v), respectively.

solubility of each drug in the feeds. According to the previous work, when CLM was spray dried alone, spherical particles were obtained (CLA). With the ionization of the dimethyl-amino sugar of CLM, using NAC as both an ionizing agent and the ionic counterpart, the formation of a corrugated surface of the spray-dried particles was evidenced by SEM images (Figure 28 E).

The increase in 2-PrOH content in the liquid feeds, however, involved the production of a blend constituted of corrugated and spherical particles. This result became more evident if the 2-PrOH content was higher, from 40:60 to 50:50, as a

result of a more rapid evaporation of the solvent during the spray drying process

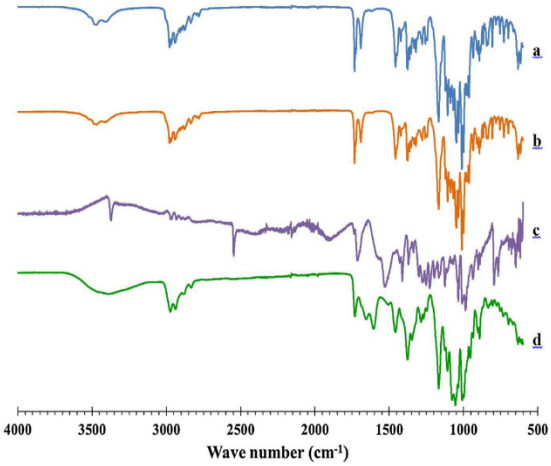

Figure 29. FTIR spectra of CLM raw material (a), CLA spray-dried (b), NAC (c), and clarithromycin-N-acetylcysteine microparticles obtained by spray drying, CLA-NAC 2 (d).

(Figure 28 H).

FT-IR studies were conducted in order to confirm the interaction between CLM and NAC in powders obtained by co-spray drying (Figure 29).

In Figure 29 the characteristic peaks of CLM crystalline raw material are reported, including the —OH stretching vibration at 3460 cm-1, the peaks between 2950 and 2800 cm^{-1} related with alkyl-CH_3 substitution bands, the peak related to carboxyl group in ketone group in the lactone ring at 1733 cm^{-1} and C=O stretching vibration from ketone group in the lactone ring at 1691 cm^{-1}, while ether function bands were observed between 1170 and 953 cm^{-1}. CLM suspension processed by spray drying demonstrated IR spectra superimposable with raw material, confirming that atomization process did not modify CLM chemical structure.

In Figure 29 is also reported the FT-IR spectrum of NAC raw material. NAC crystals showed characteristic peaks at 3372 cm^{-1} related to N—H stretching band and the S—H peak at 2548 cm^{-1} partially superposed on the broad band of —OH stretching of the carboxylic group; peaks at 1713 cm^{-1}, 1585 and 1529 cm^{-1} were related to

C=O stretching of the carboxylic group and amide I and II band, respectively (Picquart M. et al., 1998).

All CLANAC formulations exhibited a characteristic broad band centred at 3389 cm^{-1} and two peaks at 1654 and 1606 cm^{-1}, which can be associated to the interaction between the tertiary amine of the CLM and carboxylic group of NAC leading to the formation of a clarithromycin salt (Karimi K. et al., 2016).

5.3.2 Aerodynamic behaviour of CLANAC microparticles.

Gelatine capsules, commonly used for *in vitro* deposition studies, have a high moisture content compared to HPMC capsules (Richardson, 2011). Because of the hygroscopic behaviour of NAC, the present study was performed using HPMC capsules. High recovery percentages from the impaction apparatus referred to both drugs were obtained.

Furthermore, each tested powder showed excellent flow properties, evidenced by high DD values (Table 9). During the deposition study, no significant difference of FPF among #CLANAC batches was observed. Compared to #CLA, a general

Table 9.

In vitro Deposition Study using a Glass Twin Impinger (Eur. Phar. 8th). Fine Particle Fraction (FPF) and Delivered Dose (DD) of CLANAC spray-dried microparticles.

Batch #	FPF %	DD %
CLA	29.1 ± 1.0	99.9 ± 1.0
CLANAC 1	55.1 ± 2.4	100.4 ± 0.4
CLANAC 2	59.4 ± 3.1	100.1 ± 0.6
CLANAC 3	54.4 ± 4.0	100.3 ± 0.2
CLANAC 4	53.3 ± 7.1	100.1 ± 0.5

Device used to aerosolize the powders: monodose (unit-dose pre-metered in capsule) DPI RS01 model 7, breath activated DPI; Plastiape®.

enhancement of FPF was obtained for #CLANAC 2 batches (Table 9), indicating that the drug salification led to a higher aerodynamic performance.

5.3.3 Solubility assessment of CLM in CLANAC formulation.

Solubility experiments were performed in triplicate to verify if CLM ionization was accompanied by a solubility enhancement. The present study focused only on the quantification of CLM by HPLC-DAD method. The NAC solubility was not the object of this study, because this drug is very soluble in water. Furthermore, as we saw during the drugs quantification by the HPLC described method, compared to standard, HPLC-peaks of NAC were qualitatively different, indicating a probable NAC degradation, after 72 h at 37 °C.

Powders intended for inhalation are extremely fine, so the dissolution rate might seem enough rapid to have only a slight impact on the drug activity. It is well known, in fact, that a greater surface area, obtained after the spray drying process and particle size reduction, contributed to a better wettability of the microparticles. The results of this study demonstrated, however, that the solubility enhancement of CLM, approximately tenfold greater than raw material and CLA spray-dried alone, was obtained only after the ionization process (Table 10).

As reported by Hastedt et al., the dose number, D_o expressed in Equation 8 specifies a measure of the effect of the drug solubility on its dissolution process for inhaled administered drug,

$$D_o = \frac{M_0}{V \times C_s}$$

Equation 8

where M_0 is the dose, V is the volume of dissolution fluid (10 – 30 ml for the small volume of liquid lining the lungs) and C_S is the drug solubility. This dimensionless

Table 10.

Exceeded Amount of each formulation in 5 ml of phosphate buffer 50 mM, 37 °C; pH 6.75.

Batch #	Solubility of CLM (mg/ml).
CLM	0.6 ± 0.02
CLA	0.7 ± 0.02
Practically insoluble.	
CLANAC 1	11.7 ± 2.00
CLANAC 2	12.1 ± 2.40
CLANAC 3	8.7 ± 1.40
CLANAC 4	9.6 ± 0.70

index (D_o) is a very useful tool to highlight the importance of the CLM solubility enhancement when formulated as a dry powder for inhalation, evidencing the impact of drug solubility on the dissolution process. If the solubility of a drug is very low in an aqueous environment, in fact, $D_o >> 1$ and the dissolution process is impaired.

Looking at the obtained solubility data (Table 10), the solubility of #CLA is very low; therefore, the drug does not undergo to a proper dissolution process in an aqueous environment, even when formulated as microparticles ($D_o >> 1.0$, ≈ 14 for a large dose of 100 mg and a reduced dissolution volume of 10 ml). Considering the CLANAC case D_o ≈0.8 (at the same conditions), thus the enhancement of the aqueous solubility of the ionized CLM could enhance both the dissolution process in aqueous media and the possibility to administer large doses, contributing to limit

drug accumulation in the lung (Amidon G. et al., 1995; Hasted J. et al., 2016; Velaga S. et al., 2017).

5.3.4 Dissolution study of CLANAC formulation.

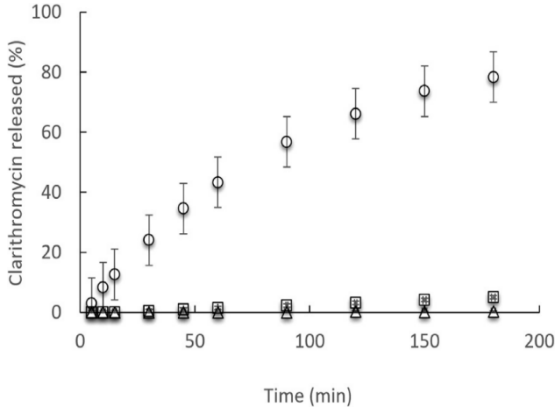

Figure 30. Dissolution profile of clarithromycin through a nitrocellulose membrane from raw material (triangles), CLA (squares), CLANAC formulation (circles).

Besides a proper deposition process that delivers the drug directly onto the lower airways, the local activity of an inhalation product formulated as a dry powder is linked to the effective ability of the drug to dissolve within the biological fluids. With their reduced volume of liquid lining their surface (10 -20 ml/100 m^2) not the ideal place for drug dissolution, and, in the case of pathology as CF, the presence of a sticky and viscous mucus further hampers the dissolution process.

In addition to the deposition studies, therefore, the dissolution test is a crucial step in the development of a formulation, as well for inhalation powders. In order to better mimic the *in vivo* environment in which CLM should dissolve after deposition, the *in vitro* dissolution studies were performed, choosing the CLANAC batch #2, for both of its higher FPF values and drug solubility. CLA (SD drug in its raw form) and CLM raw material were also preferred to make a comparison.

CLA batch (Figure 30, squares) showed again a very low drug release during time and a slow rate of dissolution, similar to CLM (Figure 30, triangles). On the contrary, we saw that the combination of CLM and NAC enhanced the wettability of the co-SD powders and increased the solubility of CLM.

As shown, without using excipients, the highest rate of dissolution was reached within 180 min, during which about 80% of the drug was released (Figure 30, circles).

5.4 Conclusions

The CLM ionization, pursued to increase drug solubility, was obtained adding in the feed solutions an equimolar amount of NAC, a well-known mucolytic drug, which could exert its action on CF lung mucus, and can facilitate the dissolution and the permeation of the antibiotic in an aqueous medium. Co-spray dried powders of CLM and NAC showed good physicochemical, technological and aerodynamic properties, appearing as a valid pharmacological support for a better management of CF respiratory disease, that combines the antibacterial activity of CLM and mucolytic properties of NAC in an optimized DPI.

Chapter 6

Part 3: MICROBIOLOGICAL EVALUATION OF CLARITHROMYCIN IN CLAHCl AND CLANAC FORMULATIONS

Experiments were carried out at:

Department of Pathology and Experimental Therapeutics;
Medical School, Molecular Microbiology and Antimicrobials.
University of Barcelona, Spain.

6.1 Scientific background and research aim

Several scientific evidences report that bacteria in nature are physically grouped in clusters and embedded by extracellular polymeric substances (Martínez L. and Vadyvaloo V., 2014). In clinical settings, pathogen bacteria can also survive naturally in aggregates when colonizing surfaces (*e.g.*, on wounds, scar tissue, medical implants) (Lindsay D. and Von Holy A., 2006; Webb J. et al., 2003), since sessile cells are less prone than planktonic ones to interact with the ordinarily used antimicrobials (Ciofu O. et al., 2015; Moskowitz S. et al., 2004; Bjarnsholt T. et al., 2013 [1,2]; Wei Q. and Ma L., 2013).

Pseudomonas aeruginosa represents one of the most resistant pathogens in CF lung disease (Jimenez P. et al., 2012); it has been often associate with a biofilm mode of growth in the lower airways and portrayed as highly recalcitrant to the antibiotic treatments (Aslam S. and Darouiche R., 2011; Flume P. and VanDevanter, D., 2015; Jimenez P. et al., 2012). Actually, lung injury and compromised respiratory functions represents the principal determinant of morbidity and mortality in CF patients (Kurbatova P. et al., 2015; Smith E. et al., 2006). Fortunately, the CF clinical treatments aimed to remove the lung colonization of *P. aeruginosa* are demonstrating advantageous and helpful to improve the life expectancy.

In collaboration with the Department of Pathology and Experimental Therapeutics; Medical School, Molecular Microbiology and Antimicrobials at the University of Barcelona, Spain, the aim of my experimental studies was therefore to evaluate, with the minimal inhibitory concentration, the maintenance of the antibiotic's activity of the CLM salt-form after a DPI formulation approach. At the same time, the behaviour of a macrolide like CLM was focused against different *P. aeruginosa*

strains (control strains and CF clinical respiratory isolates). Experiments were carried out to check the activity of CLM on the *P. aeruginosa* growth in real-time. Also, the lowest dose of CLM able to prevent a visible growth of *P. aeruginosa* from a *pre-formed* biofilm was investigated. The microbiological study will be completed by evaluating the ability of CLM batches to prevent the formation of biofilm by *P. aeruginosa* species.

On the other hand, *Staphylococcus aureus,* a Gram-positive species, represents a significant pathogen in CF especially in the childhood, that may be isolated in respiratory cultures without causing symptoms. Despite this, it may initiate an immunological response and cause significant pulmonary damage (Armbruster C. et al., 2016).

Therefore, the MIC experiments have been obtained in collaboration with the same research group, using different *S. aureus* strains.

6.2 Materials and methods

6.2.1 Bacterial species, strains used and culture conditions.

Experimental protocols and materials, concerning two laboratory strains and eight CF respiratory isolates of *P. aeruginosa*, were obtained from Department of Pathology and Experimental Therapeutics; Medical School, Molecular Microbiology and Antimicrobials at the University of Barcelona, Spain (Table 11). My work focused on the study of the antibiotic susceptibility of *P. aeruginosa* respiratory isolates towards formulated and raw material CLM.

Table 11.

Laboratory and clinical strains of *P. aeruginosa* used during the experiments.

Laboratory strains	Cystic Fibrosis respiratory isolates	
	Mucoid strains	Non mucoid strains
PAATCC 27853 PAO 1	086 SJD	056 SJD
	555.1 SJD	350 VH
	023 VH	501 VH
	639 VH	536 SJD

The CF respiratory strains were isolated from patients (sputum and pharyngeal exudates) and were collected from Hospital de la Vall d'Hebron and Hospital Sant Joan de Déu (Barcelona, Spain).

Further experiments were conducted in collaboration with the same research group to evaluate exclusively the MIC of *S. aureus* towards formulated and raw material CLM. Two control strains and three respiratory isolates and biofilm formers were used (Table 12).

Table 12.

Laboratory and clinical strains of *S. aureus* used during the experiments. In collaboration with the Microbiology Unit, University of Barcelona (Spain).

Laboratory strains	CF respiratory isolates and biofilm formers
ATCC 700689 (MRSA) ATCC 29213	SaS06 (MRSA) CLM S SaS14 (MRSA) CLM R SaS18 (MRSA)

6.2.2 Antimicrobial susceptibility of planktonic P. aeruginosa.

Antimicrobial activity of the raw material (CLM), spray-dried clarithromycin (CLA), clarithromycin plus N-acetylcysteine co-spray-dried powders (CLANAC), prepared as reported, were determined by using the broth microdilution method in Muller Hinton Broth Cation Adjusted (MHBCA).

A first liquid sub-culture of each strain was obtained from a cryogenic stock. Using a tryptic soy agar (TSA) plates, pure colonies of each strain were then cultured in a static incubator at 37 °C; after overnight, few colonies of each strain were picked and then suspended in MHBCA for an overnight sub-culture. Before starting the

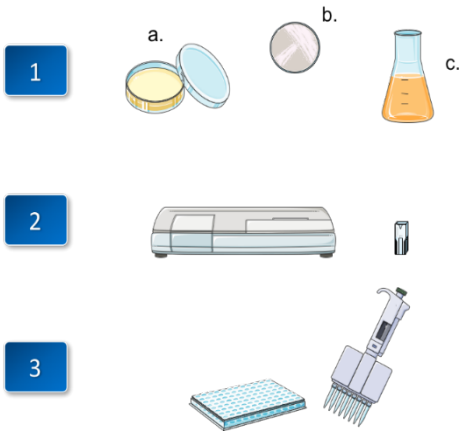

Figure 31. Steps followed to realise the MIC experiments. Step 1: (a) sawing of each strain in TSA plates; (b) evaluation of the purity of the colonies; (c) amplification of the culture in MHBCA. Step 2: determination of 10^8 colony forming unit with the spectrophotometer. Step 3: preparation of the multiwell plates to test the MIC.

experiment, the OD_{625} of the inoculum was adjusted at 0.090 ±0.005 to obtain about 1.0 × 10^8 colony-forming unit (CFU) (Thermo Scientific Spectronic, Helios Gamma) (Figure 31).

The minimal inhibitory concentrations (MICs) were determined in triplicate in at least two different experiments (n=2) by using two *P. aeruginosa* control strains – PAO1 and PAATCC 27853 and eight CF respiratory isolates, in which four strains showed a mucoid phenotype while the others were non-mucoids (Table 11). In collaboration, MIC experiments were repeated using *S. aureus* strains, in which two were control strains whereas three were respiratory isolates and biofilm formers (Table 12).

The experiments were repeated using clarithromycin and N-Acetylcysteine crystals raw materials, taken as the references (abbreviated as CLM raw and NAC raw).

All materials were dissolved in dimethyl sulfoxide (DMSO), following an adapted method.

To determine MIC values after overnight incubation, a plate reader was used to visually check the turbidity in each well.

6.2.3 Effect of clarithromycin on bacterial growth.

The growth-inhibiting activity was evaluated in real-time using clarithromycin raw material (CLM), spray-dried raw material (CLA), and formulated clarithromycin plus N-acetylcysteine co-spray-dried powders (CLANAC). All materials were firstly solubilized in DMSO and then added to 1.0 × 10^8 CFU logarithmic-phase liquid

Figure 32. Biosan® Bioreactor used to obtain the growth curves in real-time.

sub-cultures.

A control strain (PAATCC 27853) and two CF clinical isolates, a mucoid and a non-mucoid samples, (086 SJD and 056 SJD respectively; Table 11) were used. Before starting the experiment, the OD_{625} was adjusted at 0.090 ±0.005; afterward the inocula were incubated for at least 12 h in RTS-1C real-time cell growth loggers at 37 °C and 2000 rpm. Four growth curves (n=2) were described for each strain – a growth kinetic was carried out without antibiotic treatment (control) and, in the other

three loggers, the concentrations of 1/2MIC, MIC, and 2MIC were tested. The growth kinetics was measured as optical density (850 nm) in real-time (Biosan SIA, 2017. Figure 32).

The CLANAC formulation was dissolved also in purified and sterile water (Milli-Q, Millipore), following the method described above, to obtain a new set of growth curves for each strain.

Bacteria were exposed to the antibiotic for at least 12 h throughout the experiment (n=2).

6.2.4 Antimicrobial activity – planktonic vs. biofilm cultures.

Minimal biofilm eradication concentrations determinations (MBECs) were performed as reported elsewhere (Sans-Serramitjana E. et al., 2016) to evaluate the lowest concentration of CLM that prevents the visible growth of bacteria from a *pre-formed* biofilm matrix developed in a Calgary Biofilm Device (n=2). (Innovotech, 2017).

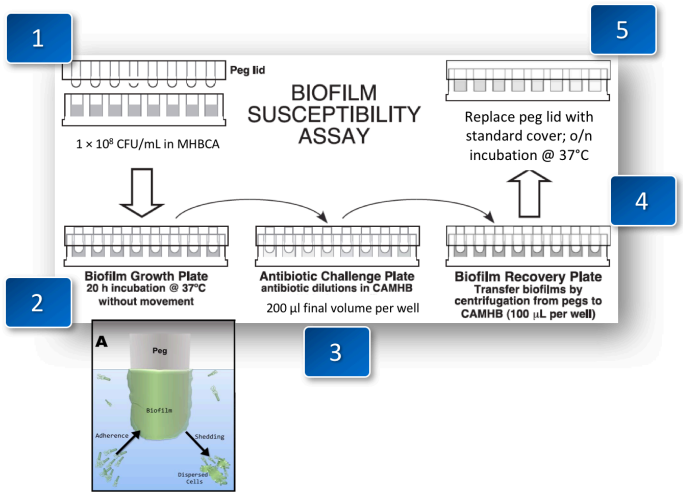

Figure 33. Minimal biofilm eradication concentration (MBECS) assay. Adapted from Moskovitz et al., 2004.

Briefly, PAO1, taken as a control strain, and two clinical isolates, the mucoid *P. aeruginosa* 086 SJD and the non-mucoid 056 SJD, were used. An overnight sub-culture of each strain was obtained; afterward, the OD_{625} of the fresh inoculum was adjusted to obtain about 1.0×10^8 CFU.

Bacterial biofilms were obtained by immersing the ninety-six (96) peg lid (Nunc-TSP; ThermoScientific, Denmark) into the 96-well microtiter plates containing 200 µl of MHBCA (NunclonTM Delta Surface; ThermoScientific, Denmark), followed by incubation at 37 °C for 24 h with no movement. The surface of the peg is constituted of a modified polystyrene to permit the formation and the attachment of the biofilm matrix around the immersed portion of the peg into each well. After overnight, the pegs were then gently washed in a diluted 1/4 Ringer's solution (1/4R) for 2 minutes to remove the not attached materials.

The peg lid with the established biofilm was subsequently immersed in the antibiotic challenge plate exposing the biofilm to increased concentrations of the antibiotic. To realize the stock suspension/solution, both crystals of CLM (raw material) and CLA (spray-dried micronized raw material) were previously

suspended by a vortexer and then homogenated by a short cycle of sonication in an aqueous solution of 10% v/v DMSO, whereas CLANAC and NAC raw material were promptly solubilized in the same solvent using only the vortex mixer.

After overnight incubation, the pegs were rewashed with a 1/4R solution for 2 min; then the established biofilm was removed by centrifugation (10 min; 2000 rpm). Recovered bacteria were incubated overnight at 37 °C in a final 96-well microtiter plate in MHBCA.

To determine MBECs values after overnight incubation, a plate reader was used to visually check the turbidity in each well of the final evaluation plate (Figure 33, step 5).

The assay was further implemented with the Minimal Bactericidal Concentrations (MBC) determination to check the dose of CLM able to prevent the growth of the colonies, and the subsequent formation of the biofilm matrix. From each well of the microtiter plate, 10 µl of medium were removed and transferred to make a spot plate on TSA plates. Afterwards, the lowest concentration of an antimicrobial producing a visible reduction of the initial inoculum of a planktonic culture was evaluated.

6.3 Results and discussion

6.3.1 Formulations and raw materials tested.

In detail, the antibacterial experiments reported below were conducted using:

- CLM (clarithromycin crystals raw material),
- NAC raw (N-acetylcysteine raw material), both taken as the references.
- CLA (micronized spray-dried raw material).
- CLAHCl (spray-dried microparticles, with salified CLM using few amounts of HCl 1M);
- CLANAC (spray-dried microparticles, with salified CLM using an equimolar amount of NAC).

6.3.2 Susceptibility of planktonic P. aeruginosa towards CLM.

Antimicrobial activity of spray-dried clarithromycin raw material (CLA base), clarithromycin plus N-acetylcysteine co-spray-dried powders (CLANAC), prepared

Table 13.

Susceptibility of *P. aeruginosa* towards raw material and formulated clarithromycin (n=2).

MICs (μg/ml)	Control strains		Mucoid strains				Non mucoid strains			
	ATCC 27853	**PAO1**	**086 SJD**	**555.1 SJD**	**023 VH**	**639 VH**	**056 SJD**	**350 VH**	**501 VH**	**536 SJD**
CLM	256	256	128	> 256	256	256	256	> 256	> 256	> 256
NAC RAW	-	-	-	-	-	-	-	-	-	-
CLA	256	> 256	128	> 256	> 256	256	256	> 256	> 256	> 256
CLA HCl	> 256	> 256	128	> 256	> 256	256	256	> 256	> 256	> 256
CLANAC	256	256	128	> 256	256	256	256	> 256	> 256	> 256

The defined MIC values, concerning free CLA against *P. aeruginosa*, occurred comparable to the described values reported elsewhere using both clinical and laboratory strains.

as reported, were determined by using the broth microdilution method in Muller Hinton Broth Cation Adjusted (MHBCA) ("Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by agar dilution," 2000). The MICs were determined in triplicate in at least two different experiments (n=2) by using two *P. aeruginosa* control strains – PAO1 and PAATCC 27853. Eight CF respiratory isolates were also used, in which four strains showed a mucoid phenotype while the others were non-mucoids.

The experiment was repeated using clarithromycin and N-acetylcysteine crystals raw materials, taken as the references (abbreviated as CLM and NAC raw). All materials were dissolved in dimethyl sulfoxide (DMSO), following an adapted method. To determine MIC values after overnight incubation, a plate reader was used to visually check the turbidity in each well.

As shown in Table 13, using two *P. aeruginosa* control strains (PAO1 and PAATCC 27853), similar values of MIC were obtained, demonstrating that the manufacturing process (spray drying and salification) did not affect the antibiotic activity.

Moreover, the reported MIC values for free CLM against *P. aeruginosa* were comparable to the described values reported elsewhere, using both clinical (Alhajlan M. et al., 2013; Hoffmann N. et al., 2007) and laboratory strains (Buyck J. et al., 2012).

As shown by the experiment, the activity of the formulated/not formulated CLM was confirmed, whereas when the clinical strains were tested, a MIC higher than 256 μg/ml was found. This evidence could be probably ascribed to the acquired adaptation mechanisms developed against antibiotics (*e.g.*, through the up-regulation of the active efflux pumps) (Moreau-Marquis S. et al., 2008; Tseng B. et al., 2013).

However, we checked a visibly lower value of MIC for the selected tobramycin-resistant and mucoid strain, 086 SJD, isolated from a young CF patient with a chronic infection (Table 13).

6.3.3 Susceptibility of planktonic S. aureus towards CLM.

As shown in Table 14, all tested formulations showed MIC values similar to the raw material form of CLM. The obtained results were related to the MIC value of erythromycin (ERM), a well-known old macrolide and precursor of the semisynthetic CLM (Table 14).

The bioactivity against *S. aureus* clinical isolates was again evidenced. This observation provides us a positive information about the different formulations of CLM dry powders (CLAHCl and CLANAC). Thus, the micronisation and salification

Table 14.

Susceptibility of of *S. aureus* species towards raw material and formulated clarithromycin (n=2).

MICs (μg/ml)

	Control strains		Clinical isolates		
	ATCC 700689 (MRSA)	**ATCC 29213**	**SaS06 (MRSA)**	**SaS14 (MRSA)**	**SaS18 (MRSA)**
CLM	> 256	≤ 0.125	0.5	64	64
NAC RAW	-	-	-	-	-
CLA	> 256	≤ 0.125	0.5	64	16
CLA HCl	> 256	0.5	≤ 0.125	64	64
CLANAC	> 256	≤ 0.125	≤ 0.125	64	32
ERM	> 32	1	0.5	> 32	> 32

of CLM after the spray drying process do not adversely affect the antimicrobial activity of the drug against planktonic *S. aureus* (MRSA).

6.3.4 Effect of formulated CLM against the P. aeruginosa growth.

The experiment was carried out by enclosing a control strain *P. aeruginosa* ATCC 27853 (Figure 34a and 34b, column 1) and two clinical strains: the non-mucoid 056 SJD (Figure 34 and 34b, column 2) and the mucoid and tobramycin-resistant 086 SJD strains (Figure 34 and 34b, column 3).

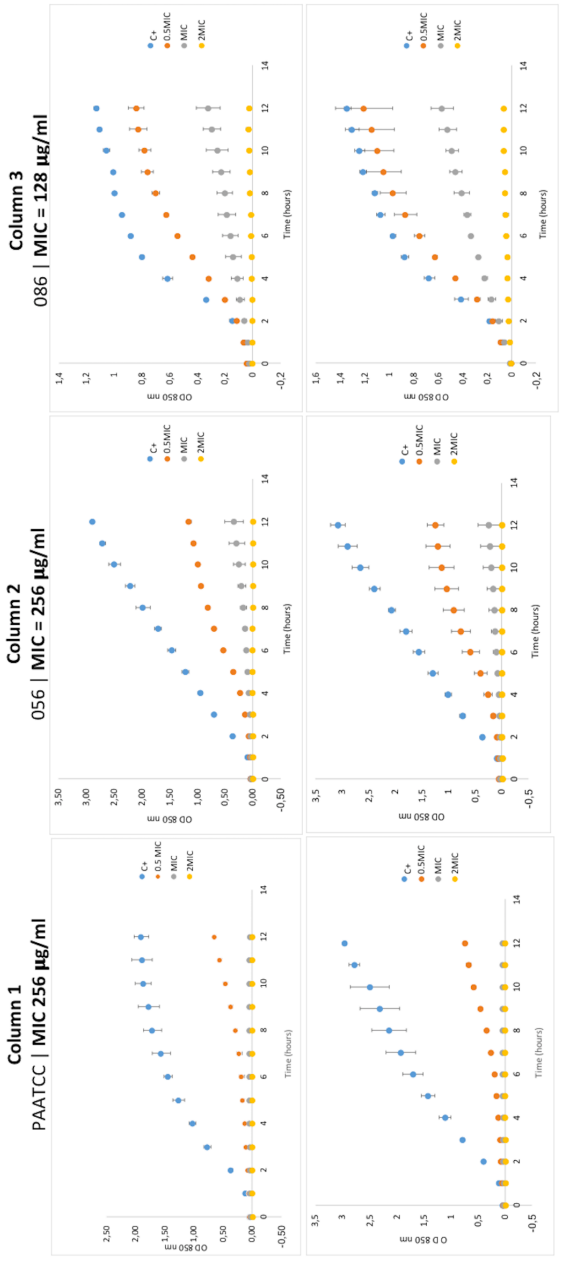

Figure 34a. Growth curves of *P. aeruginosa* strains in presence of CLM powders dissolved in DMSO (line a: raw material CLM; line b: micronized raw material (CLA) .

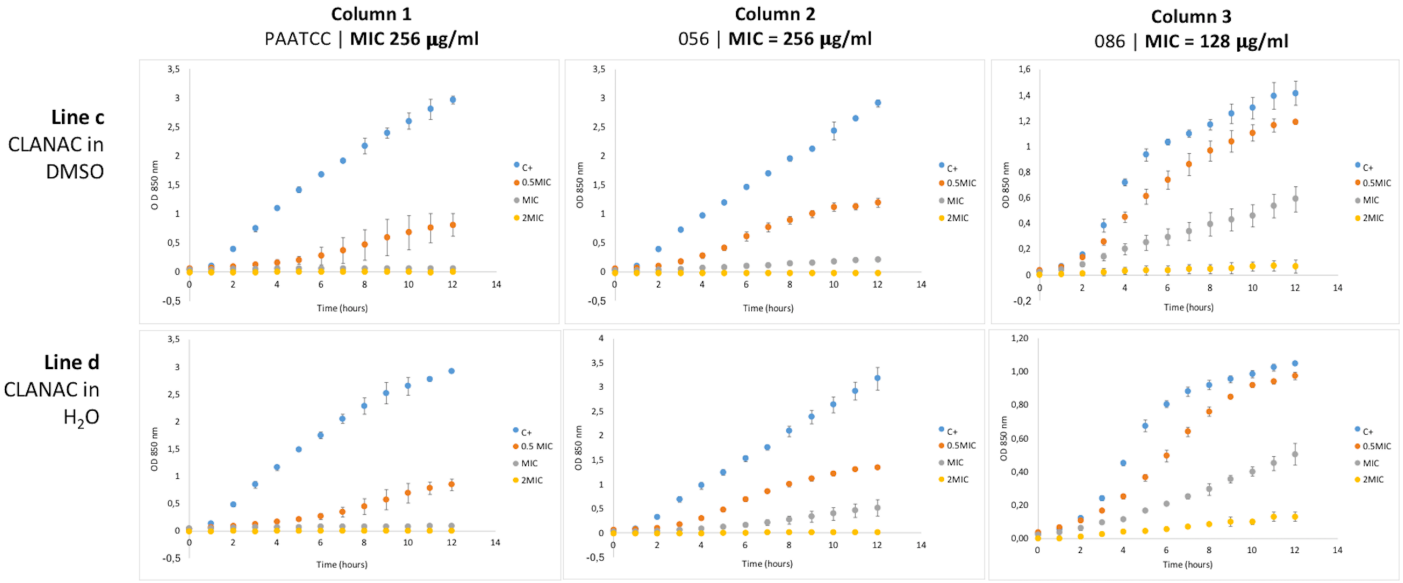

Figure 34b. Growth curves of *P. aeruginosa* strains in presence of CLANAC formulation. Line c: CLANAC dissolved in DMSO; line d: CLANAC dissolved in purified and sterile water.

As reported, for PAATCC 27853 and 056 SJD, the MIC-dose was able to inhibit the growth of the bacteria up to 12 h for all the tested powders (Figure 34a and b, column 1 and 2). Differently, for the mucoid strain 086 SJD the growth-inhibiting activity was evidenced only at the 2MIC dose (Figure 34a and b, column 3).

Therefore, in each case and within the initial five hours, bacteria incubated with the antibiotic produced an initial phase of adaptation compared with the control inoculum (antibiotic free), so the growth kinetics confirmed the activity of the formulated antibiotic (Figure 34a and b).

Moreover, the CLANAC spray-dried powder was characterized by a higher water solubility of CLM (about 10 mg/ml compared to CLM Raw). Thus, the growth curve experiments were repeated for CLANAC powders, using purified and sterile water (Milli-Q; Millipore) as solvent, instead of DMSO. The results reported in Figure 34b, line d were overlapped to the Figure 34b, line c (DMSO).

In contrast, for the CLM (raw material) and CLA (spray-dried raw material), it was not possible to run the experiment using water as solvent due to an initial turbidity of the sample which interfered with the optical measurements.

6.3.5 Antibiotic activity of CLM in the eradication of P. aeruginosa from a pre-formed biofilm.

Experiments were carried out to check the lowest dose of CLM able to prevent a visible growth of *P. aeruginosa* from a *pre-formed* biofilm (Moskowitz et al., 2004). Bacterial biofilms were obtained by immersing a ninety-six (96) peg lid (Nunc-TSP; ThermoScientific, Denmark) in the 96-well microtiter plates, containing 200 µl of MHBCA (NunclonTM Delta Surface; ThermoScientific, Denmark), followed by

incubation at 37 °C for 24 h with no movement. To realize the stock suspension/solution, both CLM crystals raw material and CLA (spray-dried micronized raw material) were previously suspended by a vortexer and then homogenated by a short cycle of sonication in an aqueous solution of 10 % DMSO. Instead, CLANAC and NAC raw material were promptly solubilised in the same solvent composition, using only the vortex mixer.

For the control strain PAO1, for the non-mucoid strain, 056 SJD and the mucoid strain 086 SJD, the highest dose of CLM was tested, but it was not able to eradicate

Table 15.

Final Evaluation of Minimal Biofilm Eradication Concentrations.

MBECs (μg/ml)

	Control	Mucoid	Non Mucoid
Strains	**PAO1**	**086 SJD**	**056 SJD**
CLM	> 1280	> 1280	> 1280
CLA	> 1280	> 1280	> 1280
NAC RAW	-	-	-
CLANAC	> 1280	> 1280	> 1280

bacterial cells from a *pre-formed* biofilm (Table 15).

The assay was further implemented with the Minimal Bactericidal Concentrations (MBC) determination (Table 16). From each well of the microtiter plate, 10 µl of

Table 16.

MBCs (μg/ml)

	Control	Mucoid	Non Mucoid
Strains	**PAO1**	**086 SJD**	**056 SJD**
CLM	> 1280	> 1280	> 1280
CLA	> 1280	> 1280	> 1280
NAC RAW	-	-	-
CLANAC	> 1280	1280	> 1280

The lowest concentration of an antibiotic producing a 99.9% CFUs reduction of the initial inoculum of a planktonic culture.

medium were removed and transferred onto the TSA plates. Afterwards, the lowest concentration of an antimicrobial producing a visible reduction of the initial inoculum of a planktonic culture was evaluated.

As shown in Table 16, CLM crystals, poured in each well as a coarse suspension, did not show a bactericidal activity because turbidity remained in each well. This evidence was attributed to the original hydrophobicity of CLM, that visibly aggregated in a precipitate throughout the overnight incubation.

However, no growth was observed for 086 SJD, treated with CLA (micronized spray-dried raw material) and for PAO1 and the mucoid 086 SJD after treatment with the CLANAC solution. This evidence could be probably linked to the higher aqueous solubility and to a homogenous distribution of the drug that characterises the ionised CLM within the dry powder formulation.

6.4 Conclusions

Hence, we can conclude that a macrolide like CLM, characterised by a well-known bacteriostatic activity, as confirmed by the MIC results for all the tested powders, was however not able *in vitro* to eradicate the bacterial cells in a *pre-formed* biofilm. In other words, combining sessile *P. aeruginosa* cells and the standard MBEC assay we did not see the killing effect.

However, a very important issue was reached concerning the improvement of the aqueous solubility of CLM, because it gave the possibility to investigate the antibacterial activity of the DPI CLM formulation, mimicking the aqueous condition of an *in vivo* environment.

Moreover, the MIC experiments reported above, that has been repeated with different *S. aureus* strains, showed an encouraging information about the different formulations of CLM.

The microbiological study will be completed in collaboration with the Department of Pathology and Experimental Therapeutics, University of Barcelona, Spain, by evaluating the ability of CLM batches to *prevent* the formation of biofilm by *P. aeruginosa* species.

GENERAL CONCLUSIONS

The thick mucus that characterises the CF airways constitutes an important barrier to the dissolution of inhaled drugs. Therefore, the stasis of the altered visco-elastic secretions allows to create a favourable environment for bacterial infections.

The dissolution and permeation properties through CF sputum of ketoprofen lysinate from a previously developed Dry Powder Inhaler (DPI) herein reported show that the CF sputum, a fluid with a pseudo-plastic behaviour characterized by a more elastic than viscous component, acts as an important barrier to drug dissolution/permeation, even in the case of an active compound very soluble in water.

In addition, by means of rheological and permeation studies, another improvement in the knowledge about the role of sodium bicarbonate in mucin expansion process was showed, because the *in vitro* addiction of a sodium bicarbonate solution (100 mM, 50 μl) to the CF sputum decreased the high viscosity of CF bronchial secretions. This statement, potentially, could result in an *in vivo* better mucus clearance.

A common, but also a beneficial symptomatic treatment of CF, is represented by the administration of antibiotics, with the aim to improve the respiratory functions.

The elaboration of an unconventional pharmacological management, like the broad-spectrum macrolide antibiotic clarithromycin, could represent an alternative proposal in the antibiotic multi-therapy management to counteract the progression

of the CF-related pulmonary worsening and to slow down as much as possible the onset of the pulmonary worsening.

Therefore, the second part of the study focused on the development of a new antibiotic dry powder for inhalation. Results showed that a fine tuning of the spray dryer parameters and of the liquid feed composition allowed to obtain powders with a good yield (up to 73.0%), suitable aerodynamic properties (FPF up to 59.4%) and increased water solubility of the macrolide clarithromycin. Thus, no excipient was necessary.

The change in pH of hydro-alcoholic solution from 10.5 to 6.5 was the breakthrough resolution to achieve not only aerodynamic properties suitable for the powder deposition in the deep lung, but also a better dissolution of the drug into the liquid lining the lung.

The CLM ionization, pursued to increase drug solubility, was also obtained adding in the feed solutions an equimolar amount of NAC, a well-known mucolytic drug. Compared to other molecules that can salify the basic group of CLM, the NAC presence in a dry powder for inhalation product could exert a mucolytic action, and can facilitate the dissolution and the permeation of the co-spray-dried antibiotic within the CF lung mucus. Co-spray dried powders of CLA and NAC showed good physicochemical, technological and aerodynamic properties, appearing as a valid pharmacological support for a better management of CF respiratory disease, that combines the antibacterial activity of CLM and mucolytic properties of NAC in an optimized DPI.

A very important issue was reached concerning the improvement of the aqueous solubility of CLM, because it conduced us to investigate the antibacterial

activity of the DPI CLM formulation, mimicking better during the microbiological experiments the aqueous condition of the *in vivo* environment.

Finally, the CLM powders were tested to evaluate several aspects of the macrolide antibiotic activity. Through the minimal inhibitory concentration assay (MIC), and the further evaluation of the growth kinetics of the CF respiratory isolates of *P. aeruginosa,* it was confirmed that the antibiotic's activity of CLM salt-form was maintained when formulated as a DPI.

However, a further conclusion to be stated is that the well-known and confirmed bacteriostatic activity of the macrolide like CLM, was not accompanied *in vitro* by the ability to eradicate the bacterial cells in a *pre-formed* biofilm. In other words, combining sessile *P. aeruginosa* cells and the standard MBEC assay we did not see the killing effect.

Further studies have been repeated with different respiratory isolates of *S. aureus*, that could be representative of an initial widespread bacterial infection in pulmonary CF disease. This first set of MIC experiments, conducted in collaboration, reported an encouraging evidence about our different CLM formulations, allowing to display a potential resource in the antibiotic arsenal to manage the early CF pulmonary infections. Also, the initial results obtained with *S. aureus* could contribute to realise an unconventional strategy in the prophylactic anti-staphylococcal management therapy, with the aim to reduce the CF clinical symptoms of the pulmonary disease.

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