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Novel respirable powder formulation: design, aerosolization and permeation studies through pulmonary epithelial cell line and mucus models

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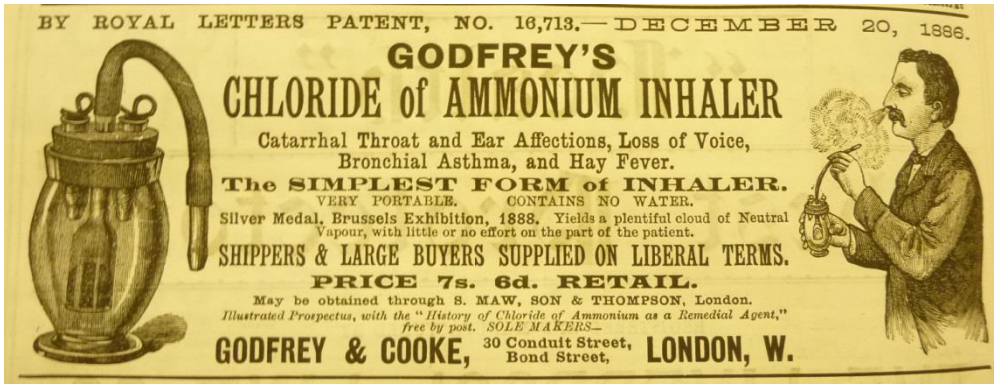
THE PhD PROJECT: AIM AND OUTLINE

Direct administration of drugs to the lungs has been used for millennia as a major treatment for a number of diseases. Origin of the inhalation therapies can be found 2000 B.C in India, where people were used to smoke *Atropa belladonna* leaves to suppress cough.



Evidence of the use of medication for inhalation dating back to 1886.

In 1986, researchers at Genentech Inc. (San Francisco, USA) discovered that the hormone of the human growth was naturally absorbed into systemic circulation of rats after its instillation into their lungs. Thus, the development of new inhalation medicines for both local and systemic administration raised a growing interest of academic and industrial researchers in the last 30 years. The first breakthrough for the treatment of a chronic systemic disease *via* inhalation was the inhaled insulin (Exubera®, Pfizer, New York, USA), available in USA from 2006 to 2007, then withdrawn from the market for economic reasons. The idea that serious diseases, such as diabetes, could be treated by pulmonary administration was going to be abandoned, until FDA (2014) decided to approve a new form of inhalable insulin, Afrezza® (Sanofi and MannKind, USA), obtained by a synergy of an innovative inhaler device Dreamboat™ and the Technosphere® technology.

Although pulmonary route is currently being exploited in ways never imagined before, local pulmonary drug delivery remains the preferred route for the administration of drugs to treat lung diseases, including tuberculosis, asthma, COPD and Cystic Fibrosis (CF).

A drug administered by the pulmonary route directly targets the airways with minimized systemic side effects, rapid pharmacologic response and reduction in the required dose. Traditional inhalers, namely MDIs (Metered Dose Inhalers), incorporate a propellant into the formulation, which provides the energy for aerosolization upon actuation. The MDIs major drawback is the need that patient must well coordinate both inhalation and actuation. Solvent- and propellant-free DPIs (dry powder inhaler) are breath-actuated, hence removing the coordination requirement above. Moreover, it has to be noted that an inhaler must 1) allow powder dispersion upon inhalation at reasonable flow rates, 2) have flow-rate-independent performances. As the dry powder formulation and the device have to be intrinsically linked to obtain a unique inhalation product, DPI is considered one of the most complex pharmaceutical product.

It is well known that a good deposition into the lung requires particles with an aerodynamic diameter in the range 1 to 5 μm. Different technologies are available to successfully produce inhalation medicines with desirable characteristic, including particle shape, size, adhesiveness, morphology and roughness. However, no standardized methods and correlated regulatory requirements are available to predict the fate of particles after the lung deposition. Hence, the old concept of pulmonary drug delivery, which states that "efficient aerosol generation and particle deposition in the lung are the main and only challenges for effective inhalation therapy", is no longer valid (Ruge *et al.* 2013).

The lack of standardized methods for the dissolution testing hinders a complete knowledge of the processes occurring after particles deposition in

the respiratory tract. The Biopharmaceutics Classification System (BCS) established by Amidon and co-workers (Amidon *et al.* 1995) for the gastrointestinal absorption, predicting the *in vivo* pharmacokinetics of the drugs, is not transferable to pulmonary case. Lung administration requires an *ad hoc* study taking into account the lung specific biology (metabolism, clearance, mucus and surfactant) as well as the characteristics of formulation and solubility of drugs, as those parameters affect the pulmonary bioavailability.

Moreover, in some pathologies, such as cystic fibrosis (CF), the presence of a thick viscid mucus may reduce the efficacy of the inhalation therapy. Thus, the study of drug–mucus interaction is a crucial step in CF to check the ability of the drug to penetrate and distribute through airways surface fluids.

In the last decade, the Research Group in Pharmaceutical Technology of the University of Salerno has been involved in developing new dry powders for inhalation. Currently, the Group has active projects in this area addressing topics such as development of DPIs containing antibiotic, anti-inflammatory and antioxidant drugs. In this frame, the aim of the present PhD project was to design inhalable powder-based formulations that could improve the treatment of pulmonary diseases, mainly cystic fibrosis. Then, the first step of the project was to formulate in a respirable form the Ketoprofene lysine salt, a nonsteroidal anti-inflammatory drug (NSAID) using the well-known Mini Spray Drying and the innovative Nano Spray Drying technology, and to evaluate limits and strengths of these different techniques. Moreover, the research focused on *in vitro* assays to evaluate the aerodynamic behavior through the respiratory system of the produced powder, using the *monodose* DPI as device for the powder aerosolization.

In the second part of the project, an *in vitro* method based on Franz-type diffusion equipment was proposed to predict the fate of drugs after deposition and to study drug dissolution/permeation processes. Moreover, to better mimic

the pulmonary environment, permeation properties of the drug were evaluated through artificial and/or native CF mucus layer. To this purpose, a mucus model was prepared taking in account physico-chemical composition and rheological behavior of CF bronchial sputum.

The final part of the project was performed at the Woolcock Institute of Medical Research in Sydney, under the supervision of Professors Daniela Traini and Paul M. Young. The research was aimed to study permeation processes of several antibiotics across Calu-3 cell line to obtain key information for the future formulation of inhaled products.

Specific objectives of the project were:

- *i)* design and development of Dry Powder Inhalers containing Ketoprofene lysine salt micronized powders by Mini and Nano spray drying production; *ii)* optimization of the aerodynamic characteristics of the powders, through the use of selected and safe excipients (amino acids) able to improve the powder flow properties and dispersion which, in turn, may increase lung deposition of the drugs (SECTION A).
- *i)* optimization and development of a model of Cystic Fibrosis artificial mucus for the permeation experiments; *ii)* rheological characterization of Cystic Fibrosis mucus patients; *iii)* permeation studies of developed formulations through both artificial and native CF mucus (SECTION B).
- investigation of the correlation between physico-chemical properties of different antibiotics, such as molecular weight, solubility, LogP and calculated permeability and their transport across Calu-3 cell line (SECTION C).

INTRODUCTION

PULMONARY DRUG DELIVERY

1.1 Pulmonary drug delivery: general background

Drug delivery to the lung is undoubtedly an effective strategy for the treatment of chronic respiratory diseases, but it is gaining substantial interest as an alternative route for the administration of systemically acting drugs that are poorly absorbed from the gastrointestinal tract or are metabolized liked polypeptides, proteins, genes or vaccines (Stegemann *et al.* 2013).

Concerning local therapy, respiratory diseases such as asthma, cystic fibrosis, chronic obstructive pulmonary disease, take advantages from direct administration of drugs to the lung in form of micronized droplets or solid particles. The benefits of local lung delivery includes rapid clinical response and minimized systemic toxicity due to reduction of overall required doses.

On the other hand, concerning systemic therapy, the lungs are an efficient way for drugs to reach the bloodstream due to the large surface area available for absorption (~100 m^2), the very thin absorption membrane (0.1–0.2 μm) and the elevated blood flow (5 l/min), which rapidly distributes molecules throughout the body. Moreover, the lungs exhibit relatively low local metabolic activity, and unlike the oral route, pulmonary inhalation is not subject to first pass metabolism (Adjei *et al.* 1994). However, pulmonary administration presents the difficulty of achieving efficient and regular deposition of the drug in the bronchial or alveolar regions of the respiratory tree, a filter aerodynamically efficient that hinders the deposition of the drug.

Moreover, the deposition and the bioavailability of the Active Pharmaceutical Ingredients (API), depend on both the formulation and the device that ensures the delivery of the correct dose of drug. Therefore the inhaled product has to be considered one of the more complex of all the pharmaceutical forms, because it is formed by an active and a special device which must be developed together (Friebel *et al.* 2012).

1.2 Anatomy and physiology of the respiratory system

To fully understand the issues related to the administration of drugs *via* inhalation, it is important to consider the anatomic and physiologic aspects of the respiratory system. The latter is formed by the nasal cavity, pharynx, larynx, trachea, bronchi and lungs. The upper airway include the nasal cavity, pharynx and associated structures, while the lower airways consist of the larynx, trachea, bronchi and lungs. The human respiratory tract presents itself as a branched system of channels for the air with more than 23 bifurcations from the mouth to the alveoli, resembling an inverted tree with a single trunk. The most widely used morphological model to describe the lung structures was initially given by Weibel who divided the respiratory tract into four regions (Weibel 1965) (Fig. 1). The upper respiratory tract is the first region and includes the nose, mouth and pharynx. Its main feature is the heating and air humidification.

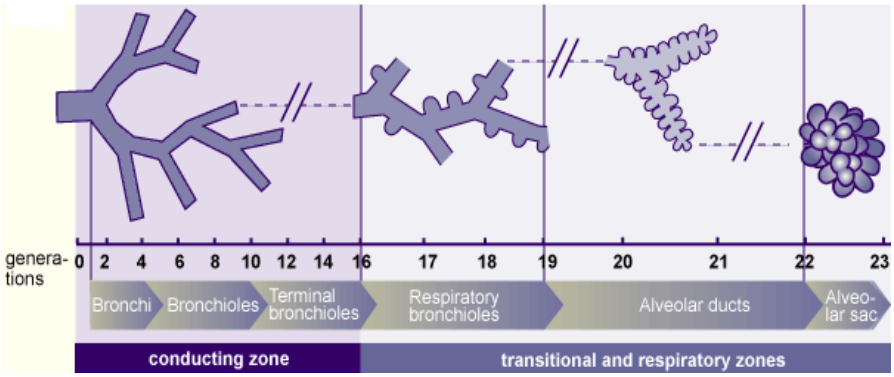


Figure 1: Airways generations.

The second region is the conduction zone which consists of the first 16 generations of branches. The transition zone is the third region that goes from generation 17 to 19. Each respiratory bronchiole consists of a few alveoli in

which occur limited exchange of gas. The fourth area includes respiratory generations 20-21-22 and 23 and ends in the alveoli.

1.2.1 Cell types lining the pulmonary airways

The entire respiratory tract is lined with a continuous sheet of epithelial cells which vary in type and function throughout the tracheobronchial tree (Fig. 2) (Gail *et al.* 1983, Jeffery 1983).

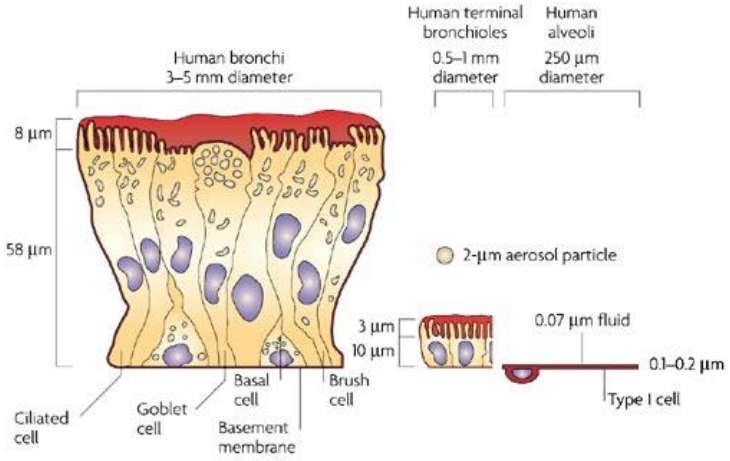


Figure 2: Comparison of the lung epithelium at different sites within the lungs.

The respiratory epithelium lining the upper airways is classified as a ciliated pseudostratified columnar epithelium.

The most prominent epithelial cells are the **ciliated columnar cells**, that line the airways from the trachea through the terminal respiratory bronchioles. From their apical surface protrude the cilia, that provide a sweeping motion of the mucus coat and play a crucial role in removing of small inhaled particles from the lungs.

Goblet cells, so named because they are shaped like a wine goblet, are columnar epithelial cells that contain membrane-bound mucous granules and secrete mucus, which helps maintain epithelial moisture and traps particulate

material and pathogens moving through the airway. They are present throughout the larger airways down to the small bronchi but are not found in bronchioles (Verdugo 1990).

Clara cells are not ciliated cells that have a spheric-shaped apical surface founded among ciliated cells. They are secretory cells and prevent the luminal adhesion, particularly during expiration by secretion of a surface-active agent (a lipoprotein) (Evans *et al.* 1986).

The alveolar epithelium is composed of a thin, non-ciliated, non-mucus covered cell layer consisting mainly of type **I pneumocytes** and type **II pneumocytes** (Gail *et al.* 1983). The first ones cover about the 95% of the alveolar surface area and are squamous. It is through type I cells that gases diffuse to allow oxygen and carbon dioxide exchange with pulmonary capillary blood. Type II cells have a more cuboidal shape and cover the remaining 5% of the alveolar surface. The main functions of type II cells are to produce pulmonary surfactant and to differentiate into type I cells after epithelial barrier injuries (Fehrenbach 2001).

Drug uptake from the lung will be affected in rate and extent both by formulation characteristics and drug physicochemical properties (partition coefficient, solubility, molecular weight). In particular, macromolecules are absorbed to a degree that is inversely proportional to their molecular weight. At this level, the mucus (1-10 μm thick) and the surfactant act as physical barriers to drug absorption, in particular in the case of peptides and protein. In addition, pulmonary enzymes deleterious for peptidic drugs are present, even though at smaller concentrations than in the GI tract. Finally, the alveoli are populated by freely roaming macrophages acting as scavengers for inhaled particles, engulfing and moving them out of the respiratory tract with their payload.

1.3 Factors affecting aerosol deposition

The inhalation product is probably the most complex of all the pharmaceutical forms: while in an oral formulation, the bioavailability depends on aspects such as solubility, dissolution rate, diffusion and stability, in the case of a pulmonary formulation, the bioavailability also requires that the dose of drug is deposited in the lower respiratory tract (Brain *et al.* 1976). A failure in deposition may result in a failure of efficacy. Since particles geometry appears so relevant to the behavior of the inhaled particles, determining the respirability of an inhalation product, the concept of **aerodynamic diameter** has been introduced to measure the size of particles to be inhaled. The aerodynamic behavior of particles depends on the so-called "aerodynamic diameter" (D_{ae}), a spherical equivalent diameter that derives from the equivalence between the inhaled particle and a sphere of unit density (ρ_0) undergoing sedimentation at the same rate (Eq. 1).

$$Dae = Dv\sqrt{\frac{\rho}{\chi\rho_0}} \qquad (1)$$

where D_v is the volume-equivalent diameter, ρ is the particle density and χ is the dynamic shape factor (Depreter *et al.* 2013). The dynamic shape factor is a correction factor that takes into account the non-sphericity of the particle: it is equal to 1 for a sphere and greater than 1 for irregular particles.

Hence, particle geometry, density and volume diameter are the main characteristics to customize since they affect inhalation performance (Buttini *et al.* 2012). It is generally accepted that particles with an aerodynamic diameter of 1-5 μm (referred to as the "respirable range") tend to deposit in the lungs, while particles larger than 5 μm are trapped in the upper respiratory tract and smaller particles (<1 μm) are exhaled during normal tidal breathing.

1.4 Mechanisms of particles deposition in the lungs

Aerosol particles entering the lungs may be deposited in the airways by several mechanisms. These include impaction, sedimentation, diffusion, interception and electrostatic precipitation (Gonda 1988). Understanding the differences in these deposition mechanisms and how they may affect the performance of formulations for inhalation is required to efficiently delivery drugs to the airways of the lungs.

1.4.1 Inertial Impaction

Inertial impaction is the dominant deposition mechanism for particles larger than 1 µm in the upper tracheobronchial regions. A particle with large momentum (large size or velocity or both) may be unable to change direction with the inspired air as it passes the bifurcations of the airways, and instead it will collide with the airway walls. Because impaction depends on the momentum of the particle, large or dense particles moving at a high velocity will show greater impaction (Heyder *et al.* 1986).

1.4.2 Sedimentation

Gravitational sedimentation is an important mechanism for deposition of particles bigger than 0.5 µm and smaller than 5 µm in the small conducting airways. It is favored by slow, deep breathing and apnea. Deposition due to gravity increases with enlarging particle size and longer residence times but decreases as the breathing rate increases.

1.4.3 Diffusion

Submicron-sized particles acquire a random motion caused by the impact of surrounding air molecules. This Brownian motion may then result in particle deposition by diffusion, especially in small airways and alveoli, where bulk airflow is very low. Therefore, to reach the lower respiratory tract and optimize pulmonary drug deposition, aerosols need to have aerodynamic diameters between 1 and 5µm (Zanen *et al.* 1996). In other words, particles larger than 5 µm usually deposit in the oropharynx from which they are easily cleared. In contrast, particles smaller than 1 µm may not deposit at all because they move by Brownian motion and settle very slowly.

1.4.4 Interception

Interception is likely to be the most effective deposition mechanism for aggregates and fibers. For such particles, deposition may occur when a particle contacts an airway wall, even though its center of mass might remain on a fluid streamline (Yeh *et al.* 1976, Gerrity *et al.* 1983, Darquenne *et al.* 2004).

1.4.5 Electrostatic deposition

Electrostatic charges enhance deposition by increasing attractive forces to airway surfaces, in particular for fresh generated particles.

1.5 Devices for inhalation products

According to the *European Pharmacopoei*a three devices can be used to release a drug in the form of aerosols in the lungs:

- Nebulizers;
- Pressurized metered dose inhalers (Dose Metered Inhaler, MDI);

- Dry powder inhalers (Dry Powder Inhaler, DPI).

This classification is based on the physical states of dispersed-phase and continuous medium, and within each class further differentiation is based on metering, means of dispersion, or design.

Nebulizer has been the first device developed for inhalation therapy market. It consists of a reservoir containing a solution or suspension of drug connected to a facemask or mouthpiece through which the patient breathes normally. The drug-containing liquid is aerosolized by ultrasound or application of compressed gas. Today nebulizers have become niche products, because of its numerous disadvantages including delivery inefficiency, drug wastage, non-portability, poor reproducibility, great variability and high cost. Moreover, aerosol administration *via* nebulization is time consuming; approximately 30 min if set-up, drug administration and cleaning are taken into account. Thus, further improvement in aerosol delivery systems with greater efficiency and portability and shorter administration time could improve patient quality of life and compliance.

In a **pressurised metered dose inhaler** (pMDI) the drug is dissolved or suspended with a propellant in a pressurized dispenser and is aerosolized through an atomization nozzle using a metering valve and an actuator (Newman 2005).

Unfortunately, there are several drawbacks associated with pMDIs. The chlorofluorocarbon (CFC) compounds formerly used as the propellant have recently been phased out as they were implicated in atmospheric ozone depletion. CFC were substituted by hydrofluoroalkanes (HFA), given its safety profile (Emmen *et al.* 2000). One of the principal limitations of pMDIs is the requirement that the patient must co-ordinate device actuation with their inhalation because of the high exit velocity of the aerosol cloud (Pauwels *et al.* 1997). Breath-actuated pMDIs and the use of spacer devices helped in reducing the issues associated with patient co-ordination.

The possibility to deliver high doses, the greater stability compared to liquid formulations and the problems releated to the use of pMDI have recently moved the research in the direction of formulating dry powder inhalers (DPIs) (Islam *et al.* 2008, Son *et al.* 2008). These devices are typically as portable and easy to use as pMDIs but since the aerosol is typically produced by the inspiration of the patient, there is no coordination issue between device actuation and patient inspiration (Cochrane *et al.* 2000, Epstein *et al.* 2001). Furthermore, DPIs do not require the use of propellants, removing the cost associated with the generation, transport, and storage of the propellant and their unwanted environmental impact. Moreover, DPIs are preferred for their stability and processing since they are typically formulated as one phase, solid-particle blends.

Currently there are essentially four types of DPIs (Islam *et al.* 2012):

- Single-unit dose (capsule) (Fig. 3); This inhaler requires the patient to load a single hard gelatin capsule containing the powder formulation into the device before each use. This is a very common type of DPI device currently available on market.
- Single-unit dose (disposable); It is a device containing a pre-metered amount of a single dose that is discarded after use.
- Multi-unit dose (pre-metered unit replaceable set) (Fig. 4); Multi-unit devices deliver individual doses from pre-metered replaceable blisters, disks, dimples or tubes.
- Multiple dose (reservoir); Multiple dose reservoir inhalers contain a bulk amount of drug powder in the device with a built in mechanism to meter a single dose from the bulk and individual doses are delivered with each actuation.

Figure 3: *Tobi Podhaler*®, single-unit dose (capsule)

Figure 4: *Diskus*®, Multi-unit dose (pre-metered unit replaceable set)

1.6 Excipients approved for use in DPIs

Owing to their small size, microparticles are extremely adhesive and cohesive resulting in low dispersibility and, consequently, poor flow properties (Chew *et al.* 2002). One way to improve the aerodynamic performance of an inhaled product is through the addition of excipients (Shoyele *et al.* 2011). Many compounds that could enhance drug delivery outcomes also have the potential to irritate or injure the lungs, so when formulating an inhalation dosage form the structural and functional integrity of respiratory epithelium must be respected (Telko *et al.* 2005). The current excipients approved by the Food and Drug Administration (FDA) for respiratory drug delivery are very limited in number and not accepted world-wide. The array of potential excipients is limited to compounds that are biocompatible to the lung and can easily be metabolized or cleared, like sugars (lactose, mannitol and glucose) and hydrophobic additives (magnesium stearate, 1,2-Distearoyl-sn-glycero-3-phosphocholine). In the last few years, amino acids (AAs) have been tested as alternative excipients due to their ability to decrease hygroscopicity and improve surface activity and charge density of particles. Different studies have demonstrated that co-spray-drying of few selected amino acids with active

compounds provides enhanced aerodynamic properties of the final dry powders (Li *et al.* 2005, Seville *et al.* 2007, Aquino *et al.* 2012).

Moreover, as amino acids are endogenous substances, they might not present a risk of toxicity to the lungs (Pilcer *et al.* 2010). Different amino acids such as arginine, aspartic acid, phenylalanine, threonine and leucine have been tested in dry powder formulations as enhancer of aerodynamic properties, the most noteworthy effects have been observed with leucine (Aquino *et al.* 2012, Depreter *et al.* 2013). For example, selection of appropriate solvent systems and leucine concentration has allowed to produce highly respirable β-oestradiol spray-dried powders (Rabbani *et al.* 2005). The influence of leucine amount on powder dispersibility and manufacturability has been reported. A 10–20% (w/w) of leucine in spray-dried ethanol or water solutions gives good aerosolization characteristics to peptides or sodium cromoglycate (Chew *et al.* 2002, Rabbani *et al.* 2005). It is suggested that addition of leucine results in less cohesive particles and in a decrease of particle size due to the surfactant behavior of leucine, reducing the size of droplets produced during atomization (Vehring 2008). Spray-dried isoleucine has also been shown to improve the aerosol performance and stability of various formulations. Trileucine has also been proven to be an efficient surface active agent able to produce corrugated particles of low cohesivity (Lechuga-Ballesteros *et al.* 2008). In this case, the stabilization mechanism seems to be different: as a result of its surface activity, trileucine molecules can orient the hydrophobic groups towards the air at the air/liquid interface during the drying process, providing a hydrophobic surface to the dry particle, thereby contributing to the observed improved aerosol efficiency.

1.7 Particles engineering

Particles engineering approaches range from traditional micronization methods to novel and sophisticated Micro- or Nano-encapsulation techniques.

As well known, traditional methods used to produce micrometric particles, such as crushing/milling and crystallization/precipitation, lead to products with a poor control of particle size, shape and morphology (Chow *et al.* 2007, Hu *et al.* 2008, Joshi 2011). Alternatively, super critical fluid drying (SCF) and spray drying are mentioned frequently in the literature (Reverchon 2002, Mosen *et al.* 2004, Pasquali *et al.* 2008) which allow to control, not only the size distribution, but also to a certain extent the particle shape and (surface) morphology (Hoppentocht *et al.* 2014).

Actually, spray drying is the most commonly technology used to generate inhalable engineered particles (Parlati *et al.* 2009, Wu *et al.* 2013).

Mini and Nano Spray drying (SD) technologies represent well know and new interesting route for particle formation, avoiding most of the drawbacks of the traditional processes.

Here are reported both the classic (mini SD) and innovative (nano SD) techniques selected to produce respirable dry powders.

1.7.1 Mini Spray Dryer

The most known instrument which uses the spray drying technology the Mini Spray Dryer (Fig. 5)

Figure 5: Mini Spray Dryer

Three basic operations can be recognized in the process of spray drying: atomization, drying and separation. The product to be dried, prepared in liquid form, is sucked from a solution and is transported to a pneumatic atomizing nozzle with a diameter of 0.5 or 0.7 mm by means of a peristaltic pump. Subsequently, the feed solution is nebulized by atomizers in small droplets in a drying chamber in which hot air circulates. The thermal contact between the spray droplets and the hot air causes the rapid evaporation of the solvent with the formation of solid particles dried or partially wet, generally spherical shape, which are then separated from the gas through a cyclone, an electrostatic precipitator or a filter (Pilcer *et al.* 2010).

The chemical composition of the solid particulates depends on the content within the feed solution, whereas particle size and morphology are strongly dependent on process parameters such as liquid and gas feed rate, inlet temperature, gas pressure and aspiration (Vehring 2008, Maas *et al.* 2011).

1.7.2 Nano spray dryer

The Nano Spray Dryer (Fig. 6) is a novel spray dryer which offers an innovative and simple approach for the production of particles from aqueous

or organic solutions, suspensions and emulsions. The technological novelty of this patented spray dryer lies in the gentle laminar drying flow, the vibrating mesh spray technology and the highly-efficient electrostatic particle collector (Li *et al.* 2010, Arpagaus 2012).

Figure 6: Nano Spray Dryer

The head is equipped with a piezoelectric crystal consisting of a thin membrane of stainless steel with an array of tiny micron-sized holes (4.0, 5.5 or 7.0 µm) (Fig. 7). An actuator moving at ultrasonic frequency (60 kHz) causes the membrane to vibrate which generates, depending on the mesh size, millions of precisely sized droplets in a size range of 3-15 µm (median diameter 5-7 µm) with narrow size distribution, mean diameter of 5-7 µm and medium size between 8 to 21 microns (Fig. 8).

Figure 7: Atomizing nozzle with a diameter of 4.0, 5.5 or 7.0 µm

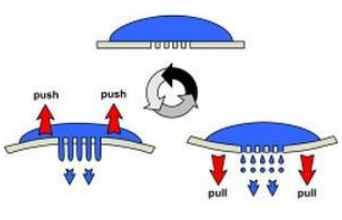


Figure 8: Working principle of the vibrating mesh

The droplets produced reach the drying chamber where, by vigorous agitation in the laminar flow of hot gas, are dried into solid particles of average size from 300 nm to 5 microns. Unlike what occurs with the Mini Spray Dryer, in which the particles less than 2 µm are not captured (Mosen *et al.* 2004), the mechanism of separation and collection of the particles in the Nano Spray Dryer is independent of the their mass and provides, due to their small size, the use of an electrostatic collector (Fig. 9). The latter consists of an internal star-shape electrode (cathode) and an external cylindrical-shape electrode (anode) between which an electric field is generated. The dried solid particles are electrostatically charged and are rejected by the cathode but attracted and collected by the anode. Hence, the particles are deposited on the inner wall of the electrode and are gently collected by means of a particle scraper.

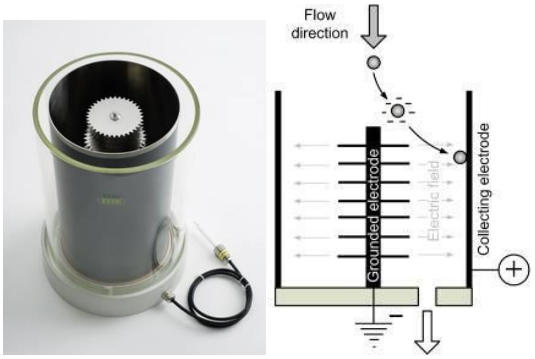


Figure 9: Electrostatic particle collector

Compared to the traditional mini spray dryer, the nano spray dryer has the following advantages:

- Production of submicronic particles or nanoparticles with a separation efficiency> 99%;
- Possibility to process even a small amount (few ml) of sample to obtain the dry powder;

- Minimum loss of product thanks to an excellent yield: up to 90% for sample quantities <100 mg;
- Process of drying quickly and efficiently with low power consumption.

CYSTIC FIBROSIS

2.1 Cystic fibrosis: general background

Cystic Fibrosis (CF) is the most common lethal inherited disease in Caucasians, occurring in approximately 1 in 3,000 newborns in the United States and Europe. The life expectancy of a child born with CF has gradually improved, but still only approaches 38 years (Elizur *et al.* 2008). CF is caused by mutations in the gene located on the long arm of chromosome 7 (7q31), containing 27 exons and which encodes the CF transmembrane conductance regulator (CFTR) (Cheng *et al.* 1990, Collins *et al.* 1990).

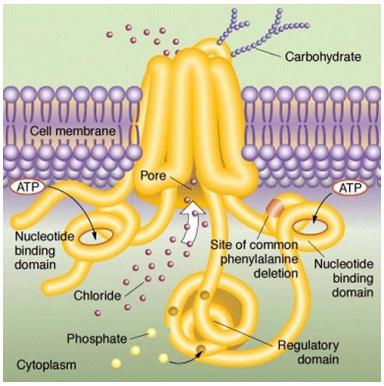


Figure 10: CFTR protein

The CFTR glycoprotein (Fig. 10), is a cyclic adenosine monophosphate (cAMP)-regulated low voltage chloride channel which is expressed in many epithelial and blood cells. It is composed of 1480 amino acids and it belongs to the ATP-binding cassette (ABC) transport protein super family. Although CFTR acts mainly as a chloride channel, it also regulates the function of other membrane proteins, including the inhibition of sodium transport through the epithelial sodium channel (ENaC) (Briel *et al.* 1998). Moreover CFTR has many other regulatory roles, including the regulation of the outwardly rectifying chloride channel, regulation of ATP channels, regulation of intracellular vesicle transport, acidification of intracellular organelles and

inhibition of endogenous calcium-activated chloride channels (Reisin *et al.* 1994, Schwiebert *et al.* 1995, Stutts *et al.* 1995, Vankeerberghen *et al.* 2002, Mehta 2005). CFTR has also an important function in the transcellular secretion of bicarbonate (HCO_3^-), an alkalizing agent that plays a crucial physiological role in pH buffering (Ehre *et al.* 2014). Reduced HCO_3^- secretion in CF may be responsible for lowered epithelial surface pH, which has been shown to impede bacterial killing (Pezzulo *et al.* 2012) and increase mucus/mucin viscoelasticity (Celli *et al.* 2005, Georgiades *et al.* 2014).

CF pathogenesis is characterized by the build-up of thick, sticky and viscous mucus which affects several organs, including the lung, the pancreas and the gastrointestinal and reproductive tracts. In particular, airway obstruction by thick mucus sets the stage for a vicious cycle of chronic bacterial infection, inflammation and airway damage which leads to progressive lung destruction and respiratory failure (Ramsey 1996). On the basis of these considerations, pulmonary disease seems to be the most challenging aspect of managing CF.

2.2 CFTR Mutations

To date, more than one thousand mutations in the CFTR gene have been identified in CF patients. The most common mutation (in 85% of CF patients) denoted as ΔF508, is a three-base deletion in the DNA sequence, causing the absence of an amino acid (phenylalanine) at position 508 of the protein sequence.

As reported in Table 1, the mutations can be classified into several different classes according to the mechanism by which they disrupt CFTR function.

Table 1: CFTR gene mutations

Class	Effect on CFTR protein	Example of mutation	% CF patients (Europe)
I	Shortened protein	Trp1282X	7
II	Protein fails to reach cell membrane	ΔF508	85
III	Channel cannot be regulated properly	G551D	<3
IV	Reduced chloride conductance	R117H	<3
V	Reduced number of CFTR transcripts due to a promoter or splicing abnormality	38949+10kb	<3

2.3 Infection and inflammation in CF airway

Lungs of CF patients are often colonized or infected in infancy and early childhood with organisms, such as *Pseudomonas aeruginosa, Staphylococcus aureus* and *Haemophilus influenza*. Among these, *P. aeruginosa* is the major pathogen in CF lung; it express specific adhesins with which binds to cell receptors that permits to settle itself into the thick mucus. Once it sets up house in the respiratory tract, is hard to get rid of. (Russo *et al.* 2013) Indeed bacterial infections lead to epithelial surface damage and airway plugging, progressively impairing airway conductance, which results in a decline in pulmonary function (Lyczak *et al.* 2002) and consequent death in cystic fibrosis patients.

Furthermore, components of the bacterial cell wall and flagella are the major activators of epithelial pro-inflammatory signaling causing a sustained inflammatory response in the lung (Fig. 11).

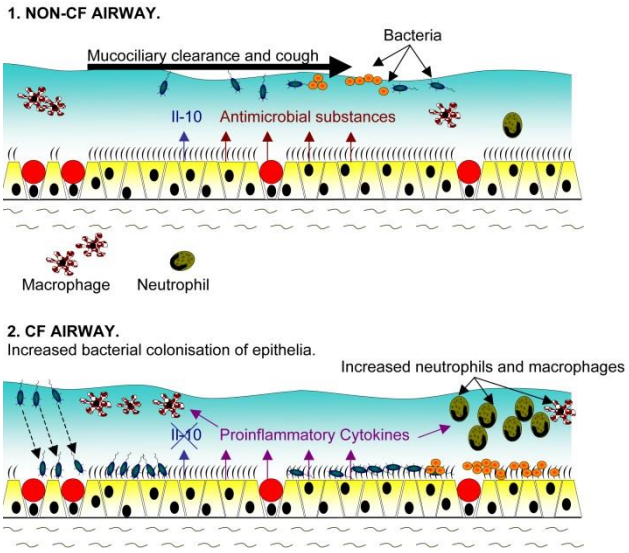


Figure 11: Infection and inflammation status in CF airways

The airway inflammation in CF appears to begin in early infancy in fact the bronchoalveolar lavage (BAL) studies from infants with CF shows high concentrations of neutrophils and pro-inflammatory mediators in the airways, even in the absence of identifiable pathogens (Nichols *et al.* 2008), indicating that inflammation can anticipate bacterial infection.

The lung inflammation is characterized by a sustained accumulation of neutrophils, high proteolytic activity and elevated levels of cytokines and chemokines (Balough *et al.* 1995, Sagel *et al.* 2007). It is also suggested that mutant CFTR may itself contribute to defective regulation of the inflammatory response in lungs (DiMango *et al.* 1995, Venkatakrishnan *et al.* 2000, Weber *et al.* 2001) and may cause a higher pro-inflammatory state compared to non-CF patients (Muhlebach *et al.* 2004). According to many researches, the airway inflammation is not only sustained, but also generated by continuous bacterial infections. This hypothesis is supported by the production of IL-8 in CF bronchial epithelial cells, in absence of an inflammatory stimulus (Venkatakrishnan *et al.* 2000). The pro-inflammatory molecular mechanisms

in the CF lung epithelium remain partially understood. The synthesis of many pro-inflammatory mediators is promoted by the over activation of the intracellular transcription nuclear factor kappa-B (NF-kB) pathway, but it is not the only pathway implicated. While the release of IL-8 by CF lung epithelial cells was highly controlled by NF-kB, other pathways such as the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) and activated protein (AP-1) pathways have been found to be involved in the lung inflammatory processes in the presence of different inductors (Li *et al.* 2003, Verhaeghe *et al.* 2007, Boncoeur *et al.* 2008). In this context, pulmonary inflammation is an important therapeutic target in CF patients, aiming to limit and delay the lung damage.

2.4 Mucus composition in CF patients

The mucus covering the airway mucociliary epithelium is generally described as a biphasic layer composed of a periciliary liquid (PCL) in which the cilia beat and a more superficial gel layer which constitutes an efficient barrier against micro-organisms (Puchelle *et al.* 2002). Normally, the mucus is constantly cleared by the action of the mucociliary transport system which serves as a fundamental, innate defense mechanism for the lung to remove pathogens and debris (Cooper *et al.* 2013).

In CF patients, the impaired electrolytic/fluid secretion caused dehydration of PLC and of the mucus layer, resulting in accumulation of dense and sticky mucus which seriously impairs the mucociliary transport and creates a favorable environment for bacterial survival and accumulation. The molecular framework and rheological properties of the CF mucus are provided by mucins and their heavily glycosylated domains which stiffen the mucin polypeptide (Gerken 1993) and provides specific ligands for bacterial adhesins (McGuckin *et al.* 2011).

Normally, pre-assembled mucin polymer are stored, condensed and dehydrate within granules of the grandular mucous cells with high concentration of Ca^{2+} and H^+ that prevent mucins expansion by neutralization of the negatives charges of mucins. Upon the exocytosis onto the epithelial surface, to promote the mucin expansion, anionic sites of mucins must be unshielded by removal of Ca^{2+} and H^+ ions, sequestered by HCO_3^- (Fig. 12).

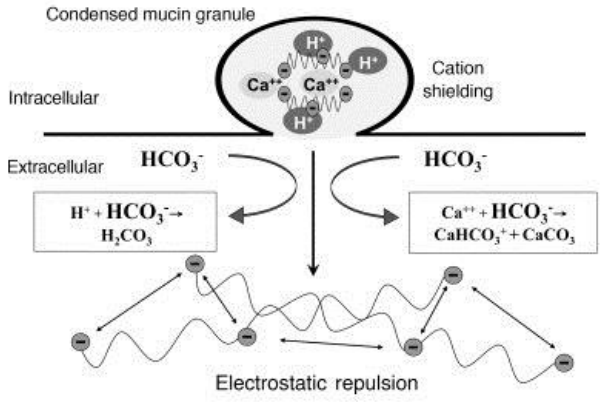


Figure 12: Possible mechanism for the expansion and solubilization of condensed mucins

In Cystic Fibrosis, defective HCO_3^-, due to a malfunction or lack of CFTR, impedes the normal hydration and expansion of mucin polymers and promotes stasis of mucus on the airway surface (Quinton 2010). The rheological properties of mucus are also influenced by alteration in lipid and DNA (released by neutrophils) contents. Girod *et al.* reported an increase in total phospholipids in CF secretions compared with normal secretions (Girod *et al.* 1992). Furthermore, DNA-actin polymers network make the mucus thicker and more rigid than normal (Puchelle *et al.* 2002, Sriramulu *et al.* 2005, Shur *et al.* 2008, Voynow *et al.* 2009). Hence, the resulting CF mucus is more viscous, tenacious and less penetrable and make the drug delivery to the lung a significant challenge for the researchers. Thus, in the case of inhalation

delivery it is crucial to study both the ability of the drug to penetrate into or pass through the mucus layer and any drug–mucus interactions which may limit the bioavailability of API (Bhat *et al.* 1996, Khanvilkar *et al.* 2001, Yang *et al.* 2011).

2.5 Current CF therapies

The understanding of the different molecular mechanisms of altered functionality of CFTR provides the scientific basis for the development of drugs for the treatment of cystic fibrosis.
Nowadays pharmaceutical research is currently proceeding on different fronts:
- Gene and cell therapy;
- Correction of pharmacological features CFTR;
- Symptomatic treatment.

2.5.1 Gene therapy

Since the discovery of the CFTR gene in 1989, CF has been considered a prime candidate for gene therapy, especially for treating the airways, since topologically they are easily accessible. Clinical trials using different gene transfer agent were conducted, but no clinical benefit has far been recorded. Gene therapy may yet provide the best solution, but the technical difficulties led to search for alternative approaches.
In vivo and *in vitro* studies on human CF nasal cells have indicated that boosting CFTR functional activity from less than 1% to as little as 5% of normal levels may greatly reduce disease severity or even eliminate the principal disease manifestations. Given the absence of other effective treatments, there has been an increasing interest in the possibility to restore the defective CFTR function by pharmacological means.

2.5.2 Modulation and correction of CFTR functionality

As reported on www.cff.org growing attention is orientated to the development of new therapies designed to modulate and/or correct the function of the defective CFTR protein according to the different CF classes of mutation (Fig. 13).

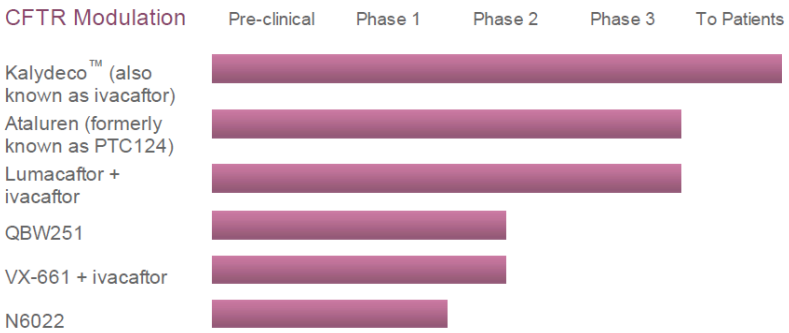


Figure 13: CFTR modulator drugs development

The first product commercially available is Kalydeco®; it is a new oral medication approved by the FDA on January 31, 2012 for people with CF ages 6 and older with the G551D mutation of CF. It aims to increase the activity of the defective channels normalizing the transport of ions through channels. Ataluren is a novel, small molecule compound that promotes the read-through of premature truncation codons in the CFTR mRNA, due to the "nonsense mutations". Lumacaftor and VX-661 are new compound, called "corrector", designed to move defective CFTR protein to the proper place in the airway cell membrane and improve its function as a chloride channel. Vertex Pharmaceuticals (Boston, USA) submitted a New Drug Application in the U.S. and Europe for the approval of lumacaftor in combination with ivacaftor for people with CF who have two copies of the F508del mutation and are 12 years and older.

QBW251 is a type of CFTR modulator called “potentiator”, similar to the drug ivacaftor, useful because it enhance the activity of normal channels already at the cell surface.

N6022 is a new injectable compound that modulates the function of the defective CFTR protein and decreases inflammation in the lung. N6022 is the first of a new class of compounds that increase levels of an important signalling molecule in the body, called S-nitrosoglutathione (GSNO). Levels of GSNO have been shown to be decreased in people with CF. These novel compounds have been shown to increase the amount of CFTR that reaches the cell membrane and to stabilize CFTR so that its function can be improved.

2.5.3 Management of Cystic Fibrosis

In the absence of drugs for gene therapy or valid correction of the basic defect, currently the most common treatment of CF is symptomatic, aimed to reduce the intensity of the symptoms, slow the progress of the disease, prevent complications and, thereby, improve the quality of life for those who suffer from this severe disorder.

Currently, CF treatment comprises respiratory physiotherapy, antibiotic therapy, nebulization with bronchodilators and mucolytic, making the pulmonary administration as one of the most effective therapies.

Antibiotic therapies

The selection of the appropriate antibiotic for CF management is done on the basis of isolation of the bacteria through the sputum culture performed periodically by each patient and based on the specific sensitivity of these bacteria in *in vitro* tests.

Different administration routes (oral, inhalation, intramuscular and intravenous) are available for the antibiotics treatment, however the inhalation route is gained growing attention in the new drug development (Fig. 14).

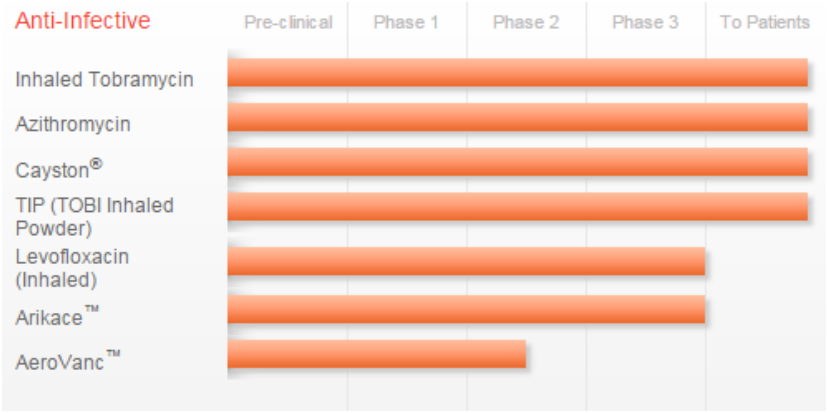


Figure 14: Antibiotics already approved and under clinical studies for the management of CF

Today, inhaled tobramycin is commercially available as two different formulation: 1) TOBI® and BRAMITOB® are an inhalation solution of the antibiotic tobramycin indicated to treat people chronically infected with *Pseudomonas aeruginosa*. 2) Tobi Inhaled Powder® is the dry powder form of tobramycin that may allow a faster, more convenient dosing regimen.

Cayston® is an inhaled form of the antibiotic aztreonam that received FDA approval in 2010 for people chronically infected with *Pseudomonas aeruginosa*. Furthermore, three different antibiotics in form of liposome or dry powder (levofloxacin, amikacin and vancomycin) are in different steps of the drug development phases.

Recently the European Medicines Agency approved Sodium Colistimethate, a polymyxin antibiotic effective against most Gram-negative bacilli, as inhaled solution (Corfinair®) and dry powder (Colobreathe®) to treat *Pseudomonas aeruginosa* infections in CF patients.

Anti-inflammatory therapies

Despite the inflammatory nature of the disease, neither oral nor inhaled corticosteroids were recommended for routine use in patients with CF, because of an unacceptable adverse event profile of oral corticosteroids, and absence of proof of efficacy for the inhaled medication (Balfour-Lynn *et al.* 2006, Flume *et al.* 2007). However, the excessive inflammatory response in the CF airways is gained growing attention of researchers and pharmaceutical companies in the developing of new anti-inflammatory products (Fig. 15).

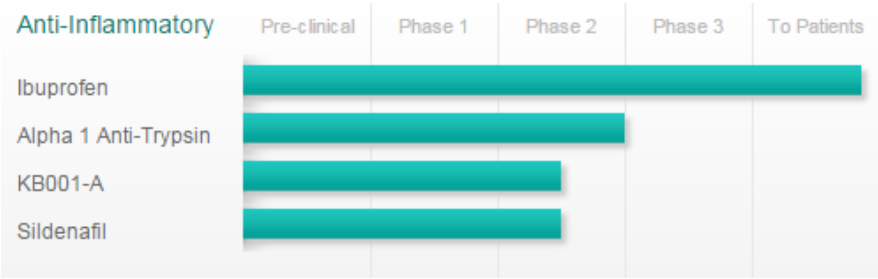


Figure 15: Anti-inflammatory approaches for the management of CF inflammation

One of the first non-steroidal anti-inflammatory drugs (NSAIDs) tested in CF was ibuprofen, able to control some of the processes involved in the inflammatory cascade. It inhibits the migration of neutrophils into the lung tissue, the production of LTB4 and the activation of NF-kB. The evaluation of the use of this drug in cystic fibrosis, its efficacy and safety in high doses, is based on data from an American four years clinical study, published in 1995 on the New England Journal of Medicine. The double-blind, placebo-controlled study, had foreseen the oral administration of high doses of ibuprofen in patients aged 5 to 39 years with mild lung disease (forced expiratory volume in one second [FEV1]> 60%). It was observed that patients treated with ibuprofen had a slower average annual decline of FEV1 (- 2.17%)

than those who were given placebo (- 3.57% per year). The beneficial effects were more evident in children between 5 and 12 years, in which the observed slower rates of decline in lung function (Konstan *et al.* 1995).

The Alpha 1 Anti-trypsin is a drug that reached the phase 2 of the clinical trial. It is an aerosolized protease inhibitor derived from human plasma and has anti-inflammatory properties because protects tissues from enzymes released from inflammatory cells, especially neutrophil elastase. KB001-A is a humanized monoclonal Fab fragment that targets a *Pseudomonas aeruginosa* virulence factor. The mechanism by which KB001-A is predicted to provide clinical benefit for people with CF is by reducing local inflammation in the lung associated with this virulence factor.

Finally, on April 2014, a phase 2 single centre trial has been completed with the purpose to determine whether sildenafil, already used in other types of lung disease, can decrease inflammation in CF lung disease.

Mucolytics agents

Today, different approaches are used to keep the mucus thin and more fluid thanks to the improvement of the movement of salt in and out of cells. Hypertonic saline as an inhaled therapy and Bronchitol® (an inhaled dry powder form of mannitol [osmotic agent]) are believed to increase hydration of CF secretions thereby improving mucociliary clearance. Pulmozyme® (Dornase alfa) is a highly purified solution of recombinant human deoxyribonuclease I (rhDNase), an enzyme which selectively cleaves DNA. It is administered as an inhaled medication with the aim to reduce the high viscosity of the mucus hydrolysing the DNA present in sputum/mucus of CF patients.

Airway clearance techniques

There are many techniques used by patients with cystic fibrosis to augment clearance of tenacious airway secretions. These methods include percussion and postural drainage, active cycle of breathing techniques, airway-oscillating devices, high-frequency chest wall oscillation devices, and autogenic drainage (i.e., chest physiotherapy in which the patient does a series of respiratory huffs and coughs designed to move mucus from distal to proximal airways so it can be coughed out) (O'Sullivan *et al.* 2009). The active cycle of breathing technique includes relaxation and breathing control, forced expectoration technique, thoracic expansion exercises, and may also include postural drainage or chest clapping.

RESULTS AND DISCUSSION

SECTION A

DESIGN AND DEVELOPMENT OF DPIs CONTAINING KETOPROFEN LYSINE SALT TO TREAT INTRINSIC INFLAMMATION IN CF PATIENTS

PART 1: MINI SPRAY-DRYING TECNOLOGY FOR THE PRODUCTION OF INHALABLE POWDERS

Based on the article: **Mariateresa Stigliani**, Rita P. Aquino, Pasquale Del Gaudio, Teresa Mencherini, Francesca Sansone, Paola Russo "Non-steroidal anti-inflammatory drug for pulmonary administration: design and investigation of ketoprofen lysinate fine dry powders". *International Journal of Pharmaceutics.* 2013, 448 (2013) 198– 204.

3A 1.1 Scientific background and research aim

Cystic Fibrosis (CF), the most common lethal monogenic disorder in Caucasians, is caused by mutations in the gene encoding the CF transmembrane conductance regulator (CFTR). Clinically, CF is dominated by chronic lung disease, which is the main cause of both morbidity and mortality. In the lung of CF patients, the inflammatory response to bacteria seems to be greater and more excessive than in normal lung (Davis *et al.* 1996, Chmiel *et al.* 2003). The lung inflammation is characterized by a sustained accumulation of neutrophils, high proteolytic activity and elevated levels of cytokines and chemokines (Balough *et al.* 1995, Sagel *et al.* 2007). It is also suggested that mutant CFTR may itself contribute to defective regulation of the inflammatory response in lungs (DiMango *et al.* 1995, Venkatakrishnan *et al.* 2000, Weber *et al.* 2001) and may cause a higher pro-inflammatory state compared to non-CF patients (Muhlebach *et al.* 2004). According to many researches, the airway inflammation is not only sustained, but also generated by continuous bacterial infections. This hypothesis is supported by the production of IL-8 in CF bronchial epithelial cell, in absence of an inflammatory stimulus (Venkatakrishnan *et al.* 2000) and by the similarity in pro-inflammatory cytokines of the bronco-alveolar lavage (BAL) of CF infants and adults (Armstrong *et al.* 2005). By contrast, in other studies (Rosenfeld *et al.* 2001, Weber *et al.* 2001, Zemanick *et al.* 2010) the BAL fluid of CF infants showed higher level of pro-inflammatory cytokines, compared to non-CF infants, even in absence of infection, thus indicating that inflammation can anticipate bacterial infection.

In this context, pulmonary inflammation is an important therapeutic target in CF patients, aiming to limit and delay the lung damage. The oral use of corticosteroids as a pharmacological treatment is at present limited to acute

pulmonary exacerbation, because of the heavy side effects experienced during a long-term administration. Moreover, corticosteroids seem to be ineffective given locally as inhalation products (Schiotz *et al.* 1983, Balfour-Lynn *et al.* 1997). Non-steroidal anti-inflammatory drugs (NSAIDs) administered orally at high doses are able to slow down the rate of deterioration of forced expired volume (Konstan *et al.* 2007) (FEV_1) and to reduce the amount of neutrophils in the lung (Konstan *et al.* 2003). However, the need of high doses of drug generates safety concerns for the long term therapy requested in CF patients. A pulmonary delivery of NSAIDs may reduce both inflammation and systemic exposure to the drug, thus avoiding its side effects. The propellant-free dry powder inhalers (DPIs), being formulated as one phase, solid-particle blends, are to be preferred thanks to their stability and easy processing. The aim of the present research was to develop respirable powders containing ketoprofen lysine salt (Klys) (Fig. 16), a well-known NSAID, and to produce a DPI able to fight the inflammatory status of CF airways by direct administration of the active pharmaceutical ingredient to the site of action.

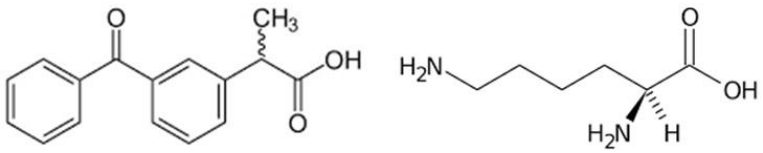


Figure 16: Ketoprofen lysine salt

Various dry powder formulations were designed, in which leucine was used as potential enhancer of flowability and spray drying as production technique. In order to fully investigate the ability of ketoprofen lysinate to reach the deep lung tissue, both the aerodynamic behavior of engineered particles and the drug permeation through a CF artificial mucus layer were evaluated. Finally, the *in vitro* effect on cell proliferation of pure ketoprofen and ketoprofen lysinate was compared to that of the engineered powders selecting CuFi1 cells

as a model of human airway epithelium of patients homozygous for ΔF 508 CF causing mutation.

3A 1.2 Manufacturing and characterization of powders

Unprocessed ketoprofen and ketoprofen lysine salt are powders unsuitable for inhalation because of low flow property and stickiness. Currently, excipients approved for respiratory drug delivery are very limited in number (Pilcer *et al.* 2010). The array of potential excipients is restricted to compounds that are biocompatible or endogenous to the lung and can easily be metabolized or cleared (Telko *et al.* 2005). Among potential excipients, recently we proposed leucine as potential enhancer of flowability and aerodynamic properties for sticky and high hygroscopic drugs (Prota *et al.* 2011, Aquino *et al.* 2012). Spray drying of acid ketoprofen with or without leucine and from different hydro-alcoholic feeds resulted in very low process yields and poor technological properties of the micronized powders. Better results were obtained starting from ketoprofen in its lysine salt form. Taking in account previous outcomes (Prota *et al.* 2011, Aquino *et al.* 2012), four different powder formulations were produced by spray drying in order to study the effect of leucine and organic co-solvent on particle formation and on their physico-chemical, aerodynamic and permeation properties. All batches were produced using the operative conditions reported in the experimental procedures (§4A 2). Particularly, Klys-1 and Klys-1a, containing only the active drug, were dried from water or 7/3 v/v water/isopropyl alcohol (IPA) mixture, respectively; batches Klys-2 and Klys-2a containing also 15% w/w of leucine, were prepared from water or 7/3 v/v water/isopropyl alcohol feeds, respectively.

Table 2. Physical characteristics of spray dried particles: liquid feed composition, process yield and particle size.

Batch	Liquid feed composition (% v/v)	Leu content (% w/w)	Yield (%)	d_{50} µm and (Span)
Klys-1	100% H_2O	-	36.4 ± 3.5	7.47 (1.54)
Klys-2	100% H_2O	15	48.0 ± 5.6	5.58 (1.72)
Klys-1a	70/30 H_2O/IPA	-	31.4 ± 5.2	4.58 (1.84)
Klys-12a	70/30 H_2O/IPA	15	78.0 ± 1.9	4.24 (1.80)

As reported in Table 2, the organic co-solvent into the aqueous feed determined a dramatic reduction of the particle size, and this effect may be explained as a decrease of the surface tension in the liquid droplets. At the same time, the presence of leucine strongly improved the process yield (up to 78% for Klys-2a), acting as enhancer of powder flowability, and contributed in reducing the mean volume diameter. Both effects combine to produce particles with a geometric diameter (D_{50}) of about 4 µm and, therefore, potentially respirable and manageable for inhalation. Moreover, considering the aerodynamic diameter formula:

$$D_{ae} = D_v \sqrt{\frac{\rho}{\rho_0 \chi}}$$

the aerodynamic properties of a powder depend on particle shape (χ) observed by means of SEM analysis (Fig. 17) besides volume diameter (D_v) and density (ρ). In particular, the shape factor is defined as the ratio of the drag force on a particle to the drag force on a volume-equivalent sphere at the same velocity (Chow *et al.* 2007). An increase in particle surface roughness corresponds to an increase in shape factor and a consequent reduction in the aerodynamic diameter.

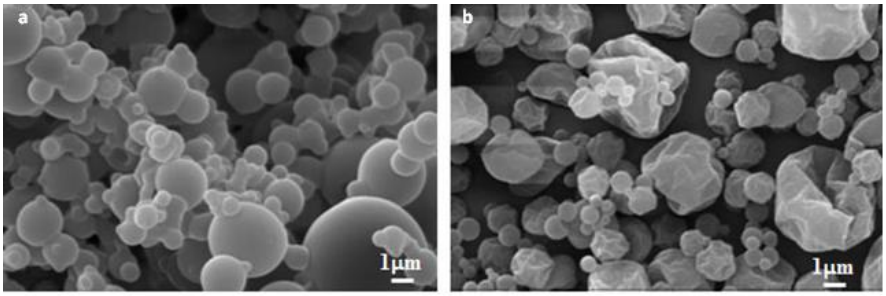


Figure 17: SEM pictures of spray-dried powders: a) Klys-1a batch containing API alone, and b) Klys-2a batch containing API and leucine as an additive.

As well known, the morphology of spray-dried particles is strongly influenced by the solubility of the components and their initial saturation in the liquid feeds. Particles containing only the active compound (Klys-1a, Fig. 17a) are smooth and spherical in shape, with a (χ) factor next to 1 and a tapped density of 0.50 g/cm^3.

According to previous observations (Lechuga-Ballesteros *et al.* 2008, Prota *et al.* 2011), during the co-spray drying process, the saturation of the lower-soluble component (leu) may increase faster than that of the hydrophilic one (Klys), due to the preferential evaporation of alcohol and the associated change in the solvent/co-solvent ratio. This led to the formation of a primary solid shell which collapsed, hence corrugated microparticles were formed.

The addition of less soluble component (leu) in the liquid feed during the spray drying process led to crumpled particles showing many wrinkles (Klys-2a, Fig. 17b) and, therefore, with increasing χ shape factor, which is likely to improve the aerodynamic performance. As reported elsewhere (Prota *et al.* 2011), powders containing leucine showed higher tapped density values (0.78 g/cm^3 for Klys-2a).

Concerning powders solid state, DSC analyses evidenced the loss of crystalline state of the drug as a consequence of the spray drying process. The thermogram of ketoprofen lysinate as raw material showed the phase transition

peak at 174.18°C (Fig. 18), whereas leucine melts at higher temperature (295.76°C).

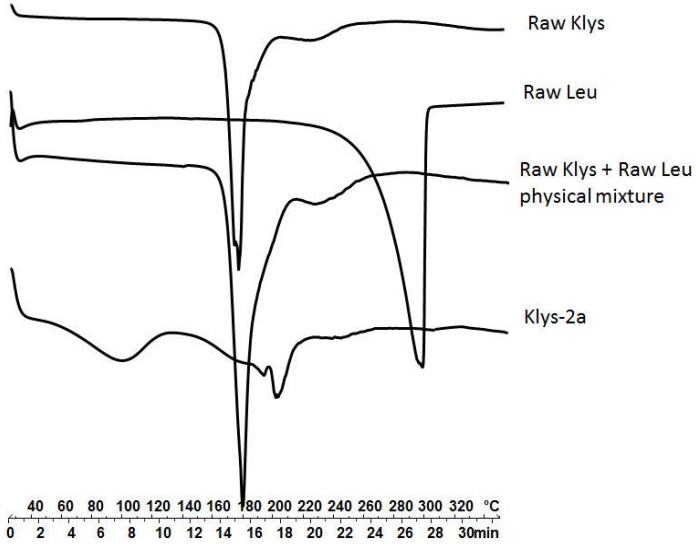


Figure 18: Differential scanning calorimetry thermograms of ketoprofen lysinate and leucine raw materials, of their physical mixture and of the spray-dried powder Klys-2a.

The absence of the endothermic peak that corresponds to the melting point of the crystalline drug in Klys-2a thermogram gives evidence of drug amorphisation occurring during the spray drying process. Moreover, in order to evidence any interaction between the drug and the excipient, a physical mixture containing ketoprofen lysinate and leucine (85:15 w/w) was prepared and analyzed by means of DSC. The thermogram of the physical mixture retained ketoprofen lysinate melting point (Fig. 18), suggesting the preservation of drug crystalline status, without evident change in the thermal behavior.

It is well known that the effectiveness of an inhalation therapy, especially for a drug powder formulation, is dependent on factors that are related to the patient and the characteristics of the formulation but also to the device (Colombo *et al.* 2013). A breath-activated, reusable *monodose* device working with a size 3 capsule was selected in the present research due to its optimal resistance rate,

ability to assure an effective particle disaggregation even with a moderate inspiration potency, as in the case of CF patients. When the capsule is pierced into the pulverization chamber by needles at the bottom and at upper side, the inhaled air creates a turbulence that shakes and twists the capsule, facilitating its empty.

Such DPI containing ketoprofen lysinate engineered powders was investigated for the ability to reach the deepest stages of the *in vitro* impactor (ACI) after emission (§4A 1.5).

Table 3: Aerodynamic properties of spray-dried powders after ACI experiments: Mass Median Aerodynamic Diameter (MMAD), Fine Particles Fraction (FPF) and Fine Particle Dose (FPD).

	Batch	**Leu content (% w/w)**	**MMAD (µm)**	**FPF (%)**	**FPD (mg)**
Water	Klys-1	-	n.d.	n.d.	n.d.
	Klys-2	15	5.28 ± 0.1	28.5 ± 1.7	8.04 ± 0.4
Water/IPA 7/3 v/v	Klys-1a	-	6.56 ± 0.43	16.7 ±1.2	6.80±1.4
	Klys-2a	15	4.39 ± 0.40	42.0 ± 3.9	11.2 ± 1.5

Results (Table 3) indicated that the aerodynamic behavior of the powders was affected by the presence of the aminoacid. Confirming our previous observations (Prota *et al.* 2011, Aquino *et al.* 2012), leucine is a convenient pulmonary excipient able to act as a dispersibility enhancer and to increase the aerodynamic parameters FPF and FPD of powders formulations for inhalation.

In fact, pure ketoprofen lysinate both as raw material or spray-dried from water (Klys-1) is unsuitable for inhalation because of the low flow property and stickiness; the powder did not come out from the capsule, hampering the aerosolization after actuation of the device (Table 3). Engineered particles

produced by spray drying ketoprofen lysinate and 15% of leucine (Klys-1a) showed improved aerodynamic properties (FPF 28.5%, FPD 8.04 mg, Table 3), thanks to the smaller diameter (Table 2) and the corrugated shape (Fig. 17). Interestingly, batch Klys-2a obtained by aqueous feed containing an organic co-solvent, achieved 42% of fine particles (FPF) that means the fraction potentially able to reach the deepest lung, after aerosolization through the *monodose* device.

3A 1.3 Cytotoxicity *in vitro* on Cufi1 cells

In order to establish whether the particle engineering may modify the toxicity profile of the drug having any cytotoxic or cytostatic effect, powders were assayed using bronchial epithelial cells bearing a CFTR F508/ F508 mutant genotype (CuFi1). CuFi1 cells were treated with increasing concentrations of ketoprofen lysine salt as raw material, Klys-1a, Klys-2a and pure ketoprofen (as control) in concentrations ranging from 0.1 to 1000 µM (expressed as ketoprofen content). The cell growth was monitored at 24 and 48 h using a colorimetric bromodeoxyuridine (BrdU) test. Results of the proliferation assay are reported in Figure 19.

Pure ketoprofen as raw material showed a faint but significant toxic effect at concentrations higher than 1µM at 48 h and then 100 µM at 24 h. Its lysine salt and spray-dried formulations showed a reduction in cell proliferation only at very high concentrations. Moreover, a significant increase in viable cells (up to 65%) was observed only for spray-dried batches containing leucine, confirming previous observations (Prota *et al.* 2011) stating that the aminoacid improves the CF cell altered metabolism, reducing the toxicity observed for unprocessed drug. Thus, the particles engineering seems to reduce the cytotoxic effect of the active compound.

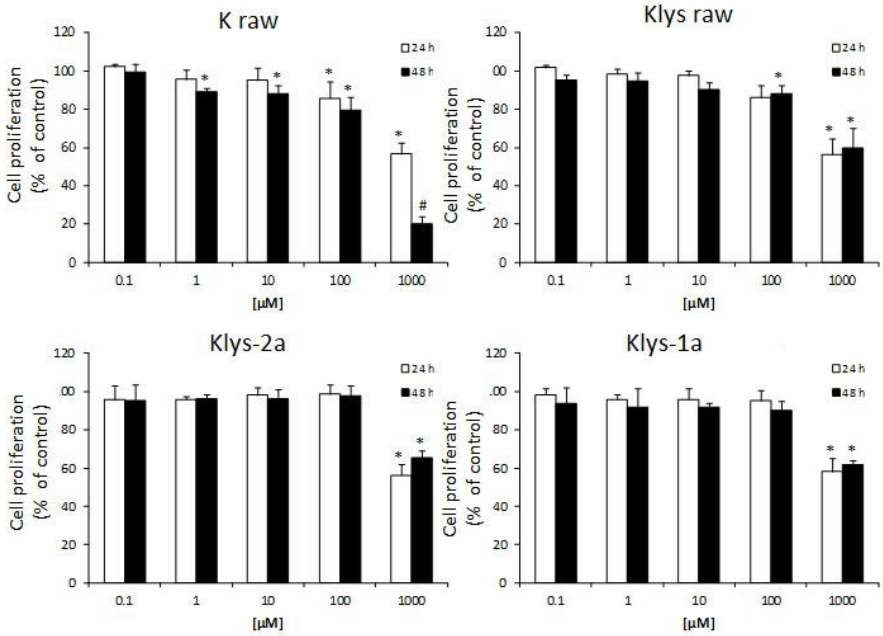


Figure 19: Effect of ketoprofen, ketoprofen lysinate and engineered powders on CuFi1 cell proliferation and viability. Cells were treated for 48 h with: neat ketoprofen, neat ketoprofen lysinate, Klys-1a and Klys-2a spray-dried at concentrations from 0.1 µM to 1000 µM (expressed as ketoprofen content). Cell viability was determined by MTT assay. All data are shown as mean ± SD of three independent experiments, each done in duplicate (*P<0.05 and **P<0.01 vs control).

3A 1.4 Conclusions

The spray drying process allowed to obtain ketoprofen lysinate amorphous powders with good process yield, low cohesivity, and reduced geometric diameter starting from hydro-alcoholic solutions and a small amount of a safe natural excipient, leucine. Micronized powders containing ketoprofen in its lysine salt form and leucine showed improved aerodynamic properties, i.e. excellent emitted dose (>98%) and satisfying fine particle fraction (up to 42%). Moreover, the particles engineering seems to reduce the cytotoxic effect of the drug on bronchial epithelial cells bearing a CFTR ΔF508/ΔF508 mutant genotype. These results make the ketoprofen lysinate based DPI a very promising product for the management of pulmonary inflammation in CF

patients. Alternative to conventional routes of administration, a pulmonary local delivery of NSAIDs may induce an inflammation control reducing the systemic exposure to the drug and, consequently, its side effects.

PART 2: NANO SPRAY-DRYING TECNOLOGY FOR THE PRODUCTION OF INHALABLE POWDERS

Based on the article: Aquino Rita P., **Stigliani Mariateresa**, Del Gaudio Pasquale, Mencherini Teresa, Sansone Francesca Russo Paola "Nano-spray drying as a novel technique for the manufacturing of inhalable NSAID powders". *The Scientific World Journal*, Volume **2014**, Article ID 838410, 7 pages

3A 2.1 Scientific background and research aim

Spray drying is a one-step process widely used to obtain a powder from a solution, suspension or emulsion, with the possibility to modulate powder physical and technological properties in relation to the specific use (De Cicco *et al.* 2014, Hoe *et al.* 2014). In particular, in the field of formulations for inhalation, the appropriate tuning of process parameters may give the possibility to modify physical properties such as powder density, particle morphology, surface and porosity, therefore dramatically influencing the aerodynamic performance for nasal and lung formulations (Russo *et al.* 2006, Dalpiaz *et al.* 2008, Prota *et al.* 2011, Buttini *et al.* 2012, Balducci *et al.* 2014). Recently, an innovative spray dryer was developed, which claimed three unique patented technologies: a laminar airflow to decrease sample loss with minimal dead volume; a spray head system to produce small particles in a very narrow size distribution and an electrostatic particle collector to obtain high yields and recover even the smallest particles (Li *et al.* 2010, Heng *et al.* 2011, De Cicco *et al.* 2014).

Differently from standard spray drying apparatus characterized by a pneumatic nozzle, in this case liquid feed droplets are generated by a piezoelectric system, vibrating a thin, and stainless steel membrane. The membrane features a series of precise micron-sized holes (spray meshes of 4.0, 5.5 or 7.0 μm hole size): after an ultrasonic frequency (60 kHz), membrane vibrates, ejecting precisely sized droplets at high speed. Moreover, the dried solid particles are electrostatically charged and collected in an electrostatic collector, so that their separation seems to be independent of particle mass as in standard cyclones and to allow collection of smaller particles; consequently, process yield may be improved (Schmid *et al.* 2011, Baba *et al.* 2012).

Among drug formulations, powders for inhalation are certainly those whose efficacy depends mainly on the particle size, strongly influencing the aerosol

deposition (Parlati *et al.* 2009, Aquino *et al.* 2012). Thus, the possibility to reduce and control particle diameter compared to standard spray drying system, makes the nano-spray drying a promising technology for the production of inhalation powders, as confirmed by recent studies (Lee *et al.* 2011, Beck-Broichsitter *et al.* 2012).

The aim of this research was to evaluate the potential of this innovative technology for the manufacturing of a dry powder for inhalation containing ketoprofen lysinate (Klys) as model drug for inflammation control in Cystic Fibrosis (CF) patients. Respirable engineered particles of this NSAID were previously prepared by co-spray drying the active pharmaceutical ingredient (API) and leucine (leu) as safe excipient. The use of aminoacid as a powder dispersibility enhancer showed no influence both on drug dissolution and permeation and on viability of ΔF508 CF (CuFi1) cells (Stigliani *et al.* 2013). With this aim we produced several ketoprofen lysinate and leucine powder batches by nano-spray dryer, studying the influence of process parameters on yield, particle properties (size distribution, morphology) and, mainly, aerodynamic properties of the resulting powders.

2A 2.1 Manufacturing and characterization of powders.

Several ketoprofen lysinate and leucine powders were produced by nano-spray drier, with the aim to evaluate the effect of different operative conditions on physico-chemical properties and aerodynamic performance of microparticles produced. On the basis of our previous research (Stigliani *et al.* 2013) in the first series of experiments Klys/leu ratio and composition of the hydro-alcoholic feed were set at 85/15 w/w and 70/30 v/v water/IPA, respectively. Powder batches and main characteristics are summarized in Table 4.

Table 4 Klys/leu powders produced by nano-spray drier: process parameters, yield, drug content and particle size distribution (15% leu and 30% IPA)

Batch	Tinlet (°C)	Total powder concentration (% w/v)	Nozzle (µm)	Yield (%)	D_{50} (µm) and ( ) span	Drug content (% w/w)	Process time (ml/min)
#1	110	5%	7	87.0 ± 2.8	6.6 (1.7)	79.4 ± 1.5	1.11
#2	110	3%	7	82.3 ± 3.2	6.2 (1.7)	77.0 ± 2.1	1.11
#3	110	5%	4	62.3 ± 2.8	3.2 (1.7)	82.9 ± 0.8	0.13
#4	110	3%	4	43.0 ± 4.2	- a	79.2 ± 3.1	0.10
#5	70	5%	4	77.6 ± 1.6	2.4 (1.7)	75.3 ± 0.4	0.06
#6	70	3%	4	56.7 ± 1.0	- a	78.5 ± 1.1	0.06
#7	70	1%	4	52.1 ± 1.8	- a	80.1 ± 1.3	0.08
#8	60	5%	4	27.9 ± 7.1	- a	73.5 ± 1.2	0.04
#9	70	5%	5.5	68.6 ± 1.9	3.1 (2.4)	72.4 ± 0.6	0.33

[a] powder stickiness prevented measure.

The first process variable considered was the dimension of spray head which at the beginning was set at 7µm, as to nozzle diameter (batch #1 and #2). Both liquid feed processed containing a powder/solid concentration from 3 to 5% w/v led free flowing powders with a very high yield (>80%) were produced; however dimensional analysis showed particles in a dimensional range expected to be not suitable for inhalation (D_{50}>6µm). Therefore, with the aim to obtained particles with a smaller geometric diameter, the 4µm nozzle was chosen, exploring the effect of both temperature and total solid concentration in the feed on powder properties (Table 4). Generally, using a 4µm nozzle, lower powder concentration (1-3% w/v, batch #4, #6 and #7) in the liquid feed led to a sticky product, difficult to handle and to test as to particle size and aerodynamic performance. Accordingly, for these powders, process yields were not satisfactory. More dense feeds, containing higher solute concentration (5% w/v of ketoprofen lysinate and leucine, batch #3 and #5)

improved process yield, producing powder in a good dimensional range (D_{50} 2.4 - 3.2µm). Moreover, it is notable that nano-spray allows to considerably reduce the inlet temperature from 110°C up to a value of 70°C, in comparison to standard spray drying technology, and this appears as a very important result for the processability of thermolabile active compounds.

Morphology study evidenced that particles where irregularly shaped but well separated from each other (Fig. 20 a, b) when produced by means of a 7µm nozzle (#2, #1).

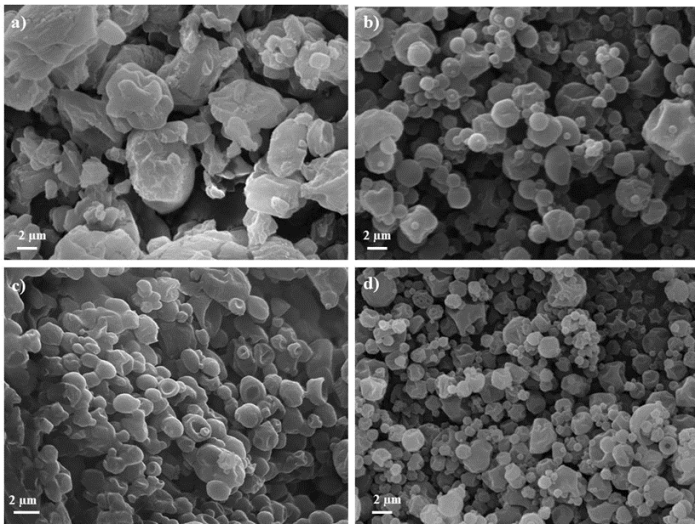


Figure 20: SEM pictures of powders obtained from a) #2 (110°C, 3% w/v, 7µm nozzle), b) #1 (110°C, 5% w/v, 7µm nozzle), c) #4 (110°C, 3% w/v, 4µm nozzle), d) #3 (110°C, 5% w/v, 4µm nozzle).

Powders obtained using a 4µm nozzle, were sensibly smaller in diameter as clearly visible in SEM picture (Fig. 20 c, d), but only the highest feed concentration (5% w/v) led to particles not agglomerated (Fig. 20 d, #3), as predictable from very low process yield and powder stickiness.

In vitro deposition behavior of non-sticky powders was evaluated using the *monodose DPI model 7* as device for the dose erogation and the Andersen Cascade as impactor. The emitted dose for all nano spray-dried batches was

almost 100% of the charged formulation, indicating a very efficient de-aggregation of powders by means of the selected device (Table 5). The dose recovered from the impactor was higher than 80% in all cases (data not shown). As expected from powders geometric diameter (>6 µm) (Table 4), batches produced by means of a 7µm nozzle showed poor aerodynamic properties, with a FPF <30%.

Table 5: Aerodynamic properties of nano-spray-dried Klys/leu (15% leu, 30% IPA) powders after ACI experiments: Emitted dose (ED) Mass Median Aerodynamic Diameter (MMAD), Fine Particles Fraction (FPF) and Fine Particle Dose (FPD).

Batch	**Feed concentration (%), nozzle (N) and temperature (T)**	**Emitted Dose (%)**	**MMAD (µm)**	**FPF (%)**	**FPD (mg)**
#1	5% N_7T_{110}	99.2 ± 0.1	5.69 ± 0.23	21.9 ± 2.5	6.27 ± 0.5
#2	3% N_7T_{110}	99.2 ± 0.1	5.44 ± 0.55	29.5 ± 2.3	8.16 ± 0.24
#3	5% N_4T_{110}	99.1 ± 0.1	4.25 ± 0.12	50.4 ± 1.9	12.7 ± 1.1
#5	5% N_4T_{70}	100.0± 0.1	3.72 ± 0.07	66.3 ± 1.0	16.9 ± 0.6
#6	3% N_4T_{70}	96.7 ± 4.2	6.25 ± 0.11	31.6 ± 1.6	7.85 ± 1.2
#9	5% $N_{5.5}T_{70}$	97.3 ± 1.0	4.58 ± 0.14	45.6 ± 2.2	12.2 ± 1.3

Among powders produced by means of the 4µm nozzle, very interesting aerodynamic properties were evidenced for batch #5 showing a FPF of 66.3%, together with the smallest geometric diameter and a high process yield (5%N_4T_{70}, Table 5).

Figure 21 shows the amount of drug deposition on the throat, on the stages 1 to 7 and on the filter, expressed as percentages of the total powder recovered from the impactor. Confirming its excellent aerodynamic performance, batch #5 was characterized by a very low deposition on the throat and onto the upper stages of the Andersen Cascade Impactor (Fig. 21).

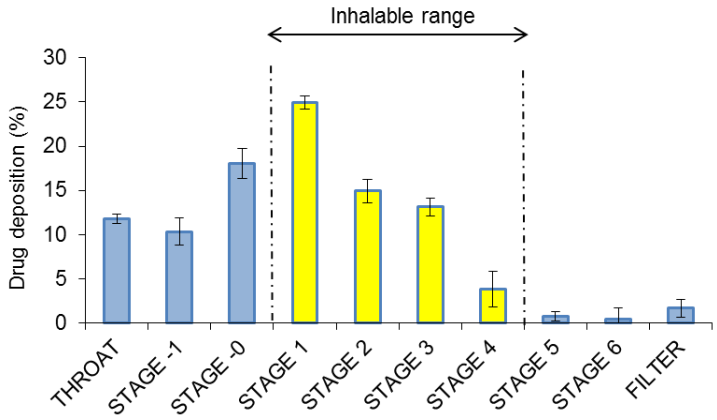


Figure 21: Andersen cascade impactor deposition pattern after the aerosolization of powder (batch #5) sprayed with a 4 µm nozzle at 70°C (#5% N4T70).

However, in addition to an improvement of the aerodynamic properties of the powders, the reduction of nozzle size from 7 to 4µm led also to a dramatic increase in process time (Table 4), moving from 1.11 to 0.06 ml/min of liquid feed processed. The portion of liquid pumped into the atomization head and not sprayed went back to the feed container in a continuous loop, exposing the sample to the inlet temperature for very long times. In the case of Klys, this effect caused the production of yellowish powders (Fig. 22).

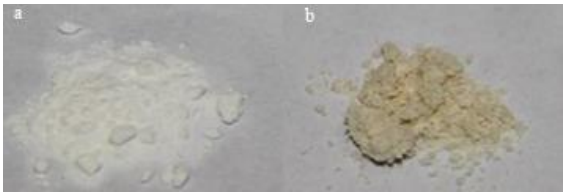


Figure 22: Picture of Klys/leu powders after nano-spray process using 7 (#1, a) and 4µm nozzle (#5, b).

Several attempts were pursued with the purpose to reduce thermal stress of the processing fluid (i.e. use of ice bath, reduction of batch volumes), with unsatisfactory results. Finally, with the aim to reduce viscosity at the nozzle and increase droplet formation efficiency, nozzle was dressed with a film of

surfactant dipping the vibrating membrane into a span 80 n-hexane solution (0.05% w/v) and allowing the solvent to evaporate. As expected, the thin film of surfactant changed the influence of process parameters on powder properties, so that a new set-up of the apparatus was necessary. The main process variables considered were total powder concentration (ranging from 5 to 7% w/v), drug/leu ratio (from 85/15 to 90/10 w/w), and water content (from 70 to 100% v/v) in the hydro-alcoholic feeds. The characteristics of the main batches prepared with the surfactant treated nozzle are summarized in Table 6.

Table 6: Process parameters and physical characteristics of Klys/leu particles sprayed through a surfactant treated nozzle (70°C, 4µm nozzle): liquid feed composition, yield and dimensional distribution.

Batch	**Total powder concentration (% w/v)**	**Leu concentration (% w/w)**	**Water/IPA (% v/v)**	**Yield (%)**	**D_{50} (µm) and ( ) span**	**Process time (ml/min)**
#5a	5%	15%	70/30	33.1 ± 9.2	-a	0.21
#5b	6%	15%	70/30	45.1 ± 3.5	-a	0.21
#5c	5%	10%	70/30	74.8 ± 2.5	- a	0.21
#5d	6%	10%	70/30	75.2 ± 1.0	3.01 (1.80)	0.21
#5e	7%	10%	70/30	18.2 ± 7.5	- a	0.21
#10	6%	15%	100/0	71.7 ± 1.2	3.14 (1.86)	0.14
#11	6%	15%	90/10	75.3 ± 0.5	2.84 (1.63)	0.42
#12	6%	15%	80/20	68.7 ± 2.6	2.94 (1.90)	0.06
#13	6%	10%	90/10	76.2 ± 3.1	2.52 (1.90)	0.21
#14	6%	10%	100/0	65.5± 1.5	3.14 (1.69)	0.10

[a] powder stickiness prevented measure.

The surfactant layer on the spray head membrane generally caused a speeding up of the atomization (up to 0.42 ml/min) and a consequent reduction in process time.

As evidenced by SEM pictures reported in Figure 23, the amount of leucine influenced particle morphology and surface properties, both for aqueous and hydro-alcoholic liquid feed processed. In particular, particles from powders containing 10% leucine were spherical in shape (Fig. 23 a,b), while particles from powders containing higher amount of leucine (15%, Fig. 23 c,d) were wrinkled and corrugated. This is a very important effect, since an increase in particle surface roughness corresponds to an increase in shape factor and a consequent reduction in the aerodynamic diameter.

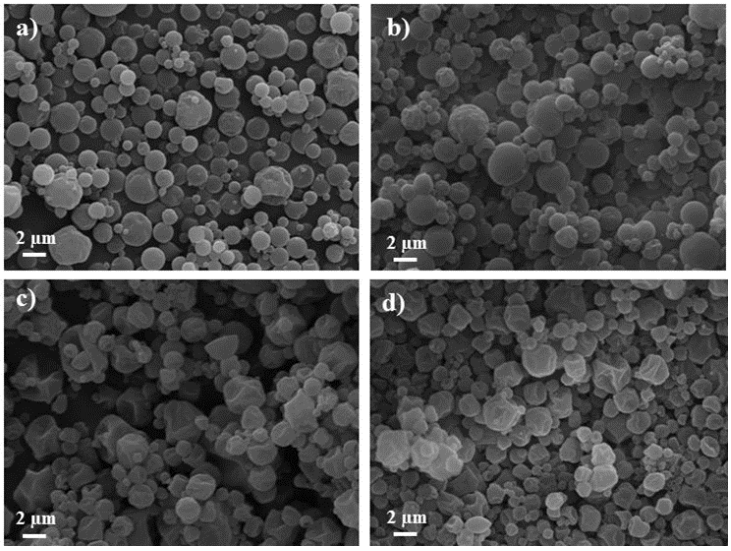


Figure 23: SEM pictures of powders obtained from a) 14# (6% w/v, Water/IPA 100/0 v/v, 10% leu), b) #13 (6% w/v, Water/IPA 90/10 v/v, 10% leu), c) #10 (6% w/v, Water/IPA 100/10 v/v, 15% leu), d) #11 (6% w/v, Water/IPA 90/10 v/v, 15% leu).

Finally, concerning powder technological properties, improvements were obtained by increasing the concentration of dry content up to 6% and/or reducing the amount of organic solvent in the liquid feeds (from 30 to 0% v/v), resulting in both cases an increase of solution viscosity. In particular, batches 11 and 13 produced from 90/10 v/v hydro-alcoholic solutions were white and free-flowing powders: among these, batch 11 containing 15% w/w of leucine,

represented the most promising product, in terms of process efficiency (0.42 ml/min of liquid feed processed and 75.3% yield).

Table 7: Aerodynamic properties of Klys/leu powders sprayed through a surfactant treated nozzle (70°C, 4µm nozzle, 6% w/v feed) after ACI experiments: Emitted dose (ED) Mass Median Aerodynamic Diameter (MMAD), Fine Particles Fraction (FPF) and Fine Particle Dose (FPD).

Batch	Leu concentration (% w/w)	Water/IPA (% v/v)	Emitted Dose (%)	MMAD (µm)	FPF (%)	FPD (mg)
#5d	10%	70/30	98.4 ± 2.9	4.25 ± 0.12	54.4 ± 0.3	15.8 ± 0.4
#10	15%	100/0	100.1 ± 0.1	4.02 ± 0.05	54.8 ± 1.1	18.2 ± 0.7
#11	15%	90/10	99.7 ± 0.3	3.83 ± 0.02	60.9 ± 1.2	18.0 ± 0.4
#12	15%	80/20	99.9 ± 0.2	4.19 ± 0.20	55.3 ± 2.5	16.6 ± 1.1
#13	10%	90/10	100.8 ± 0.1	4.31 ± 0.22	49.9 ± 3.2	15.5 ± 0.9

Besides process efficiency, DPI containing #11 presented also the highest fraction of drug with an aerodynamic diameter less than 5µm, as reported in Table 7. Increasing particle roughness and corrugation, leucine positively influenced powders aerodynamic performance, with FPF values up to 60.9%.

3A 2.2 Conclusions

The nano-spray drier system seems to be an efficient alternative to standard spray drying in formulating dry powders with reduced geometric diameter and increased aerosol performance. Certainly, the accurate tuning of process variables is necessary to allow the preparation of fine powders with physico-chemical and aerodynamic properties suitable for inhalation. In the case of ketoprofen lysinate, using the smallest nozzle (4µm) process times were considerably high, resulting in brownish powders. To reduce the process time and gain a good yield, a surfactant thin film covering the nozzle was required, with the aim to increase drug solution passage through the micron-sized holes

of the membrane, accelerating powder production. The selection of process variables allowed us to obtain a white powder with satisfying aerosol performance and able to release 18 mg of fine particles after one actuation of the *monodose* device.

SECTION B:

DRUG PERMEATION STUDIES THROUGH ARTIFICIAL CF MUCUS AND HUMAN BRONCHIAL SECRETIONS

3B Scientific background and research aim

Apart from deposition, systemic or local pharmacological activity of an inhalation product depends on drug dissolution into the biological fluids lining the lung. Consequently, during the development of a new DPI, the *in vitro* dissolution profile of the formulated drug is an essential way of anticipating its *in vivo* behavior. *Official Pharmacopeias* provide standardize methods for the dissolution test of solid or semisolid, oral or dermal dosage forms, well established both for quality control testing and for prediction of *in vivo* drug release. However, no regulatory requirements for testing medications for inhalation are available to date and this gap must be bridged (Russo *et al.* 2013). In 2008, the Inhalation *Ad Hoc* Advisory Panel of USP reviewed all current literature and, considering the strengths and limitations of the published procedures for *in vitro* dissolution testing of inhalation dosage forms, it concluded that there was no compelling evidence that dissolution was "kinetically and/or clinically crucial for currently approved" inhaled products (Riley *et al.* 2012). Nevertheless, in the recent years there has been growing interest of academic and as well as industrial researchers in the development of *in vitro* techniques to determine the dissolution profile of products for inhalation. Different apparatus have been used in published reports of dissolution testing of inhaled products, namely, USP 2 paddle apparatus, custom-built flow-through apparatus and mainly diffusion-controlled cell systems (Davies *et al.* 2003, Son *et al.* 2009).

The definition of such a standardized method is a tricky step, due to the peculiar anatomy of the deep respiratory tract. The test conditions must reflect the pulmonary environment, particularly the thin liquid layer on which the inhaled particles impact (10–20 ml/100 m^2 of surface). Moreover, in some pathologies, such as cystic fibrosis (CF), the presence of a thick viscid mucus may reduce the effective drug delivery to the target tissues. More than in other

pathologies, the study of drug–mucus interaction is a crucial step in CF, to check the ability of the drug to penetrate and distribute through airways surface fluids.

Various methods and models for estimating permeability of drugs potentially delivered as pulmonary aerosol, have been proposed e.g. Side-By-Side® diffusion apparatus (Bhat *et al.* 1995, Agu *et al.* 2011), as well as Franz type vertical dissolution cell (Donnelly *et al.* 2007) using a modified configuration to evaluate drug permeation through a mucus layer.

PART 1: PERMEATION PROPERTIES TROUGHT CF MUCUS MODEL OF GENTAMICIN AND KETOPROFEN LYSINE SALT FROM DPIs

Based on the articles:

Mariateresa Stigliani, Rita P. Aquino, Pasquale Del Gaudio, Teresa Mencherini, Francesca Sansone, Paola Russo "Non-steroidal anti-inflammatory drug for pulmonary administration: design and investigation of ketoprofen lysinate fine dry powders". *International Journal of Pharmaceutics.* 2013, 448 (2013) 198– 204.

Paola Russo, **Mariateresa Stigliani**, Lucia Prota, Giulia Auriemma, Carlo Crescenzi, Amalia Porta, Rita P. Aquino, "Gentamicin and leucine inhalable powder: What about antipseudomonal activity and permeation through cystic fibrosis mucus?. *International Journal of Pharmaceutics 440* (2013) 250– 255

3B 1.1 Research aim

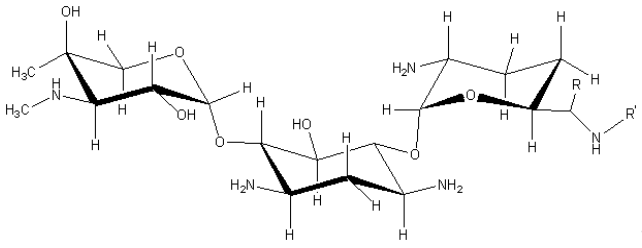
With the aim to investigate dissolution and permeation properties of dry powders for inhalation developed during the PhD project an *in vitro* method based on Franz-type diffusion equipment was proposed.

In the first part of the study the experiments were performed dusting the powder directly onto a synthetic membrane, to explore the effect of particle engineering and excipient on the drug dissolution/permeation.

Afterwards, to better mimic the CF pulmonary environment, permeation properties of the drug from DPIs were evaluated spreading the powder on a thin layer of artificial CF mucus poured on the membrane. To this purpose, a mucus model was prepared taking in account physico-chemical composition and rheological behavior of CF bronchial sputum.

3B 1.2 Permeation properties of gentamicin/leu DPIs

Aminoglycosides, such as gentamicin sulfate (G) (Fig. 24), are indicated in the management of acute exacerbations of CF as well as in the control of chronic infection and the eradication of *P. aeruginosa* infections. Moreover G has shown the ability to partially restore the expression of the functional protein CFTR (cystic fibrosis transmembrane conductance regulator) in CF mouse models bearing class I nonsense mutations (Wilschanski *et al.* 2000, Clancy *et al.* 2001, Du *et al.* 2002, Wilschanski *et al.* 2003).



$\cdot H_2SO_4$

	R	R'
Gent.C1	CH_3	CH_3
Gent.C2	CH_3	H
Gent.C1a	H	H

Figure 24: Gentamicin sulfate

In a previous study (Aquino *et al.* 2012) four out of twenty G dry powders designed and having satisfying aerosol performance were produced by spray-drying hydro-alcoholic feeds with 30% (v/v) of an organic co-solvent (isopropanol, IPA or ethanol, ET). GET3 and GISO3 contained pure G; GET3-leu15, GISO3-leu15 were manufactured with G and 15% (w/w) of leu, which was the aminoacid concentration giving rise to the best respirability parameters, as reported in Table 8.

Table 8: G dry powders: composition, yield of the spray drying process, particle size distribution and aerodynamic properties (evaluated as MMAD mass median aerodynamic diameter, FPF Fine Particle Fraction and FPD Fine Particle Dose) after Andersen cascade impactor deposition experiments.

	Batch	**Leu content (% w/w)**	**Process Yield (%)**	**D_{50} µm and (Span)**	**MMAD (µm)**	**FPF (%)**	**FPD (mg)**
H_2O/Et 7/3 v/v	GET3	-	74.8 ± 3.5	4.01 (1.82)	n.d.	n.d.	n.d.
	GET3-leu15	15	68.2 ± 2.9	4.16 (1.71)	5.3 ± 0.1	28.5 ± 1.7	8.5 ± 0.4
H_2O/IPA 7/3 v/v	GISO3	-	85.5 ± 1.1	4.24 (1.97)	6.6 ± 0.4	16.7 ±1.2	6.8 ± 1.4
	GISO3-leu15	15	82.0 ± 1.5	3.90 (1.62)	4.4 ± 0.4	42.0 ± 3.9	11.2 ± 1.5

The less polar co-solvent isopropanol led to higher process yields compared to ethanol whereas leu had no positive effect on the quantity of powder recovered from the spray drier. G and leu content in the final spray-dried powders determined by HPLC (almost 100% of the theoretical dose, data not shown), and particle size analysis by LLS (D_{50} 3.90-4.24 µm) indicated optimized process conditions. However, GET3 and GISO3, produced without leu, were highly cohesive and too sticky and, therefore, presented very poor aerosolization and deposition performance (FPF <9%), evaluated by ACI

experiments. The presence of 15% leu dramatically increased FPF (>40%) and FPD values (>47mg) (Table 8). In particular, batch GISO3-leu15 dried from water/isopropanol feed showed the best inhalation profile and the ability to deliver high fraction dose to the lung, emitting 56.4 mg of fine G after one device actuation.

With the aim to study G permeability and how the aminoacid in the drug formulation may influence G dissolution and permeation through a synthetic membrane, we selected Franz-type vertical diffusion cells equipment.

In a first set of experiments, buffer permeability was evaluated by means of Franz cells in a standard configuration (Fig. 25), spreading the powder directly on a nitrocellulose membrane in the donor compartment as described in the experimental procedures (§4B 5).

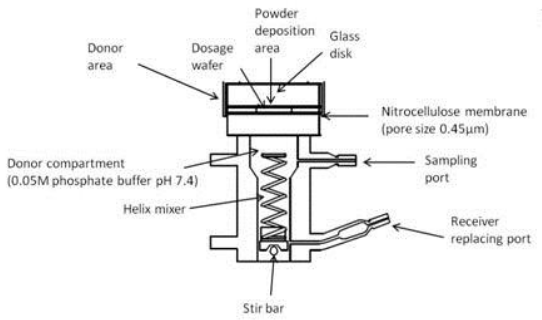


Figure 25: Franz cell used in permeation studies: a) standard configuration with one dosage wafer housing the drug formulation; b) modified configuration with three dosage wafers housing mucus and drug formulation.

Results are reported in Figure 26. As already known, drug dissolution is influenced by the rate at which solvent-drug interactions overcome the cohesive forces between bioactive molecules (Davies *et al.* 2003). Therefore, as expected, powders containing neat G, a very soluble and high polar drug, showed a typical diffusion controlled release, not influenced by the dissolution process (Fig. 26, open squares). The addition of leu, amino-acid with a lipophilic side chain, must reduce powder wettability and, consequently, the

affinity between the solid and the medium. Accordingly, the drug permeation rate from G-leu batches (Fig. 26, black squares), was reduced and this effect may be due to a slower dissolution of the drug once in contact with the wet membrane surface. No difference in buffer permeability was observed for powders dried from water/ethanol (Fig. 26a) or water/isopropanol (Fig. 26b) feeds.

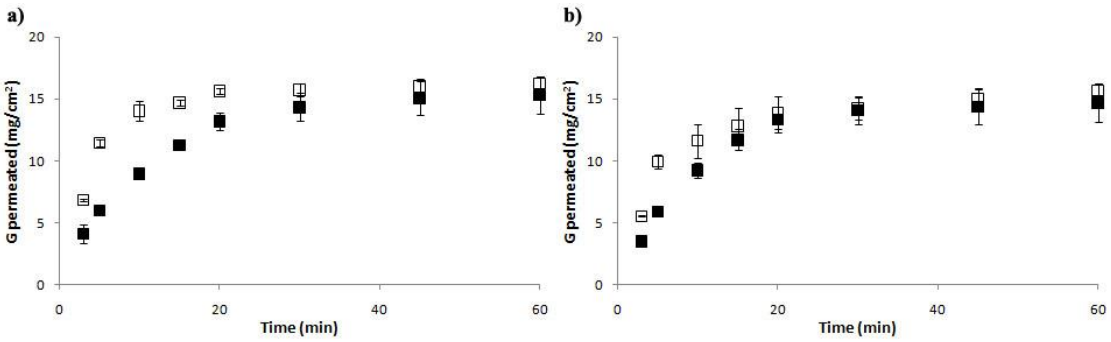


Figure 26: Buffer permeation profile of gentamicin through nitrocellulose membrane of dry powders with (GET3-leu15 and GISO3-leu15, black squares) or without (GET3 and GISO3, open squares) leucine and spray-dried from a) water/ethanol or b) water/isopropanol, 7/3 v/v.

The buffer permeability is useful to estimate diffusivity through an aqueous medium and provides a baseline for successive experiments. In order to better mimic the pulmonary environment, G permeability in a synthetic mucus layer was evaluated, too. This model was prepared taking in account physico-chemical composition and rheological behavior of CF bronchial sputum (Livraghi *et al.* 2007, Rubin 2007). The gel-forming glycoprotein mucin and other typical constituents such as DNA, salts, surfactant, lipids, proteoglycans, are considered responsible for the viscoelastic properties and able to reduce drug permeation (Bhat *et al.* 1996, Khanvilkar *et al.* 2001, Yang *et al.* 2011).
Preliminarily, we prepared a CF simplified model (AM-) as described elsewhere, (Sriramulu *et al.* 2005). However, as already noticed by other authors (Yang *et al.* 2010), the resultant gel was too fluid compared to CF

sputum specimens (viscosity ~ 2 Pa×s at 0.16 Hz) (Shur *et al.* 2008) and passed through the membrane pores during the permeation studies. Experimentally, we found no more mucus retained on the membrane surface after 60 min. Hence, aiming to increase mucus viscosity while maintaining its basic composition, the inert rheological modifier hydroxyethylcellulose (HEC 1% and 1.5% w/v) was added, obtaining AM_1 and $AM_{1.5,}$ respectively. The viscosity properties of the mucus models developed were evaluated by means of a rotational viscometer as described in the experimental procedures (§4B 3). AM_1 and $AM_{1.5}$ showed a rheological behavior typical of pseudo-plastic fluids (Fig. 27), with a viscosity reduction after shear stress increase similar to that observed for whole sputum of patients affected by CF (Shur *et al.* 2008).

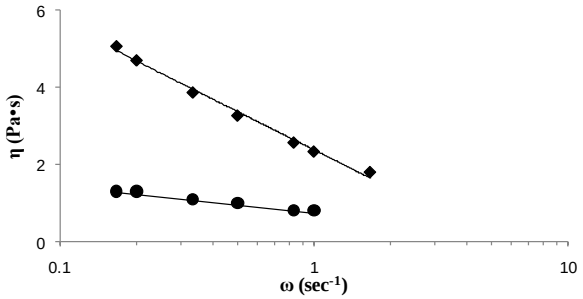


Figure 27: Viscosity values (η) versus oscillation frequency (ω) for synthetic CF mucus model AM_1 (circles) and $AM_{1.5}$ (triangles)

Both mucus samples were retained on the membrane surface throughout the experiment, without modifying aspect or viscosity during 180 min of testing and allowing the fulfilment of the permeation study.

In the second set of experiments, G permeability was evaluated using the Franz cells in a modified configuration (Fig. 28) (Donnelly *et al.* 2007) interposing AM_1 or $AM_{1.5}$ between the nitrocellulose membrane and the drug formulation as described in the experimental section (§4B 5).

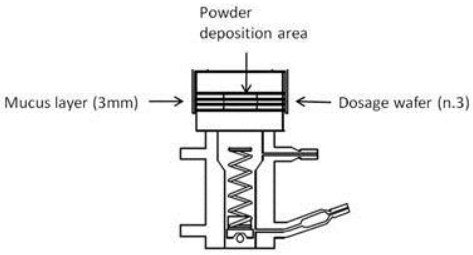


Figure 28: Modified configuration of Franz cell used in permeation studies with three dosage wafers housing mucus and drug formulation.

Results are reported in Figure 29a,c for AM_1 and Figure 29b,d for $AM_{1.5}$, (batches with leu, black squares, without leu, open squares) in comparison to buffer permeation profile (triangles).

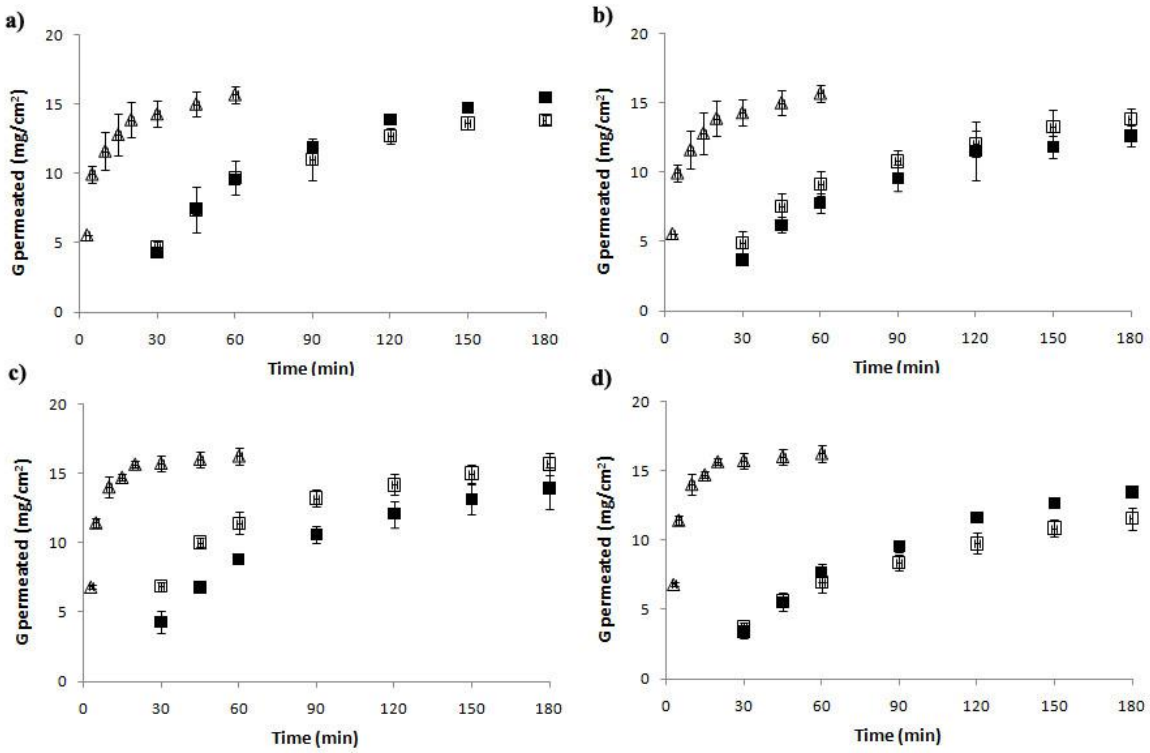


Figure 29: Mucus permeation profiles of gentamicin from powders with (GET3-leu15 and GISO3-leu15, black squares) or without (GET3 and GISO3 open squares) leucine and dried from a-b) water/isopropanol, c-d) water/ethanol and 7/3 v/v; through a-c) mucus AM_1 and b-d) mucus $AM_{1.5}$. As comparison, buffer permeation profiles of GISO3 (a-b) or GET3 (c,d) are reported (triangles).

AM_1 and even more $AM_{1.5}$ reduced significantly G permeability, compared with the corresponding buffer permeability. Moreover, leu had only a faint influence on G permeation properties. This result seems to be a key issue; the selected excipient does not interfere with the ability of the drug to permeate through a mucus layer. The observed decrease in drug transport needs to be considered for compounds that must cross a mucosal surface prior to action or absorption; however, it is an interesting property in the case of antibiotics, such as G, which must be locally bioavailable for acting against bacteria colonizing mucus layer.

3B 1.3 Permeation properties of ketoprofen lysine salt/leu from DPIs

The dissolution/permeability of ketoprofen lysine salt from the engineered particles (prepared as reported in §3A 1.2), was evaluated too, using the method based on Franz-type diffusion equipment as described in the experimental procedures (§4B 5).

Dry powder formulations were produced using mini spray drying technique. Batch Klys-1a, containing only the active drug was dried from water and Klys-2a containing also 15% w/w of leucine, was prepared from water or 7/3 v/v water/isopropyl alcohol feed.

Initially, Franz-type cells assembled in the traditional configuration were used to follow the drug permeation in the receiving compartment (phosphate buffer 0.05 M, pH 7.4) dusting the powders (Klys-1a and Klys-2a) directly onto the wet membrane. Results are reported in Figure 30. The spray-dried pure drug (Klys-1), due to the high drug solubility (water solubility: 67.7 ± 5.1 mg/ml) showed a typical diffusion controlled release, not influenced by the dissolution process. Solvent-drug interactions may easily overcome the cohesive forces between very polar and soluble drug molecules.

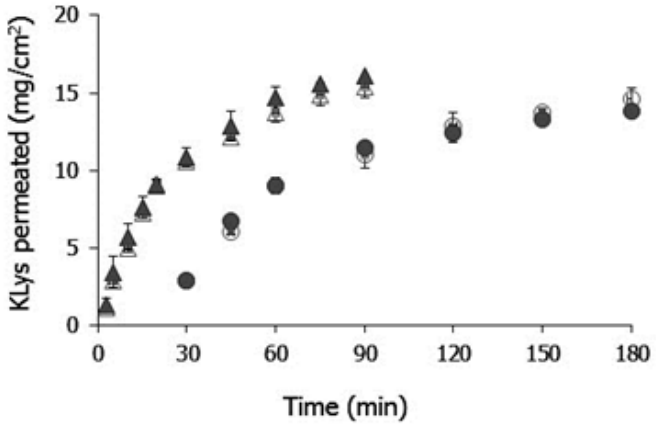


Figure 30: Buffer (triangles) and mucus (circles) permeation profiles from ketoprofen lysinate powders produced with leucine (Klys-2a; full triangles and circles) or without leucine (Klys-1a; empty triangles and circles).

The addition of a less polar additive such as leucine (water solubility 22.4 ± 0.2 mg/ml) had no effect on the drug permeation process, as evidenced by the perfectly overlapped permeation profiles (triangles) of Klys-1a and Klys-2a. In a second set of experiments, the drug permeability was evaluated interposing a mucus layer between the powder and the membrane, in order to study the effect of the mucus on the permeation properties. The artificial mucus with composition similar to that of CF patient and containing hydroxyethylcellulose as inert thickening agent was prepared as described in §4B 2.

Results reported in Figure 30 (Klys-1a empty circles; Klys-2a full circles) showed that the mucus layer slowed down ketoprofen lysinate dissolution and permeation, acting as a physical barrier to the permeation of the drug. Moreover, the presence of the aminoacid, leucine, did not influence the velocity and the amount of active compound able to dissolve and permeate into the receiving compartment through the mucus layer. It is well known that the drug contact with lung fluid is an early stage in pulmonary pharmacokinetics, before absorption or distribution can take place.

3B 1.4 Conclusions

A synthetic CF mucus and Franz-type cells may be useful to investigate drug–mucus interaction, a critical event that puts the efficacy of inhaled medications at risk. Starting from literature data, we improved a basic mucus model using an inert rheological modifier to obtain a fluid with rheological properties similar to CF mucus and able to overstay the membrane surface throughout the experiment time. The method herein proposed was applied to investigate the permeation of respirable engineered powders containing gentamicin sulfate or ketoprofen lysine salt as drug and leucine as dispersibility enhancer.

The use of the synthetic mucus in the permeation studies evidenced that the very polar active compounds formulated as a fine powder retain their ability to dissolve and permeated through an artificial mucus layer, with no influence of the excipient. Moreover the present research suggests that leucine is a very interesting additive for pulmonary use, able to enhance the aerodynamic performance of dry powders but does not affect the dissolution of the drugs.

PART 2: RHEOLOGICAL PROPERTIES OF CYSTIC FIBROSIS SPUTUM AND DRUG PERMEATION STUDY

3B 2.1 Scientific background and research aim

In CF patients, the impaired electrolytic/fluid secretion caused the build-up of thick and sticky mucus-gel responsible of both airway obstruction and loss of pulmonary function. The molecular framework and rheological properties of the CF mucus are provided by mucins and their heavily glycosylated domains which stiffen the mucin polypeptide (Gerken 1993) and elevated DNA content which can exhibit specific interactions with mucin leading to an increase of elasticity of the mucus solutions (Bhat *et al.* 1996). Moreover, many studies indicated that that CF transmembrane conductance regulator (CFTR) is required for bicarbonate (HCO_3^-) transport and that HCO_3^- is critical for normal mucus formation (Garcia *et al.* 2009, Gustafsson *et al.* 2012). Indeed, defective HCO_3^-, impedes the normal hydration and expansion of mucin polymers and promotes stasis of mucus on the airway surface (Quinton 2010).
Hence, this part of research aimed to study rheological properties of human bronchial mucus obtained from CF patients, thanks to the collaboration with *U.O.C. Genetica Medica* and *Centro Fibrosi Cistica* of Giannina Gaslini Institute of Genoa.
With the intent 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 the mucus, permeation of ketoprofen lysinate (Klys) as Dry Powder Inhaler was evaluated.
Finally, the effect of a saline solution containing high concentration of $NaHCO_3$ on sputum viscosity and drug permeation was studied.

3B 2.2 Rheological properties of CF mucus patients

For CF mucus, oscillatory rheological test was performed, which better mimic the *in vivo* stress conditions in the airways, minimizing the structural changes of the glycoprotein. Human bronchial secretions were obtained from CF patients at the "Istituto Giannina Gaslini" received as frozen samples at -20°C and maintained frozen until use. Before use, samples were softly unfrozen by placing them in refrigerator for 12 hours.

Rheological measurements were performed using an ARES rotational rheometer (Rheometrics, Inc.) with a parallel plates geometry (plate diameter 25 mm, gap of 0.5 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 by means of strain sweep preliminary measurements. Four parameters were identified dependent on frequency (ω, s^{-1}): η^* (complex viscosity), G' (elastic modulus), G'' (viscous modulus) and tan δ (ratio of G'' to G'). All samples were tested at 37°C and all experiments were carried out under air flux.

For rheological tests the only gel fraction of the CF mucus was loaded onto the plate. The gel phase was separated from the liquid phase either by means of centrifugation (10 minutes at 11000 *g*) or by simply lifting it with a spatula, allowing the liquid phase to drip off.

After the method setting-up, measurements on three mucus specimens (#1, #2, #3) coming from three different CF patients were conducted.

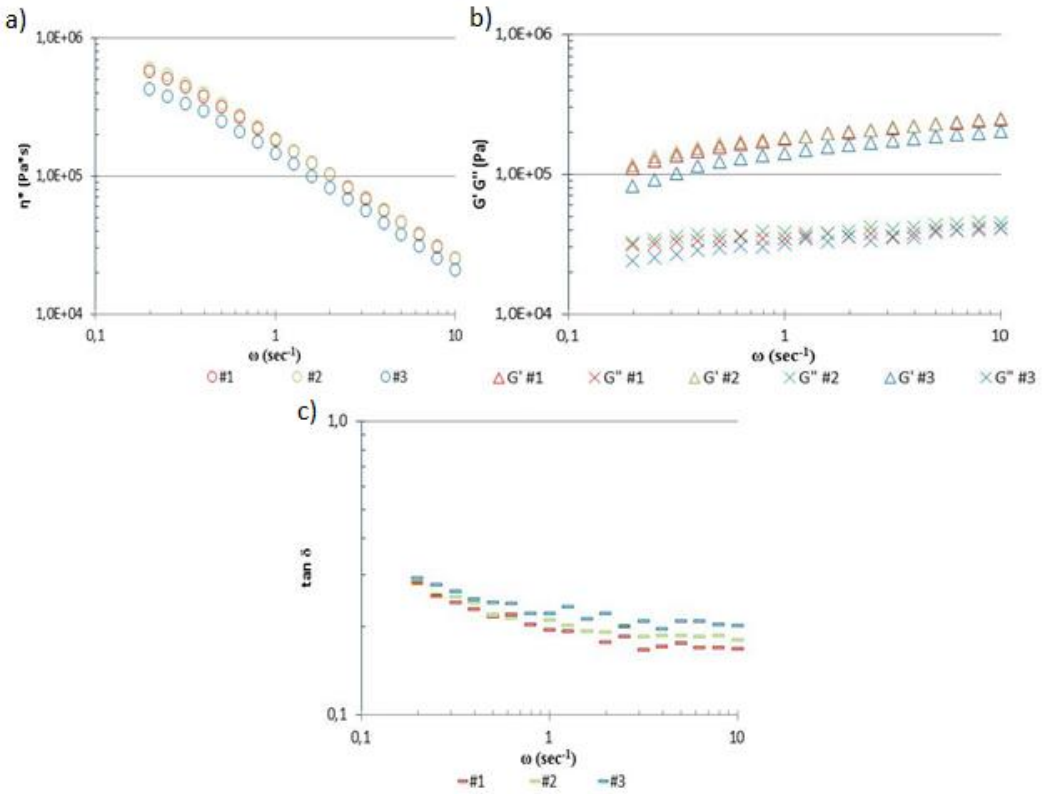


Figure 31: Rheological parameters of three mucus samples versus oscillation frequency (ω); a) complex viscosity b elastic and viscous modulus) c) Tan δ

As reported in Figure 31a complex viscosity decreased with increasing frequency, as for pseudo-plastic fluids, where the macromolecular components of the material align themselves in the directions of the applied force, reducing their resistance to the flow. The elastic moduli (G') was always greater than the viscous moduli (G'') (Fig. 31b). Moreover, G' and G'' increased with frequency. Tan δ values (Fig. 31c) are sensibly less than 1 (G'>G''), indicating an elastic behaviour higher than a viscous one. 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. These preliminary results my explain the retention of the mucus in the airway even though the cilia movements: if the material is

elastic, it will return to its initial shape and size when these forces are not exerted any more.

On the basis of the previous considerations regarding the role of HCO_3^- in the physiological expansion of mucin, rheological studies were repeated on 1 ml of untreated mucus (W, added with 100 µl of water) and on 1 ml of mucus obtained from the same patient after the same specimen collection and treated *in vitro* with 100 µl of $NaHCO_3$ (CARB, final concentration in the mucus ~100mM),. Samples were introduced in an incubator shaker at 37°C for 30 minutes, in order to allow the mixing of water or bicarbonate with the mucus.

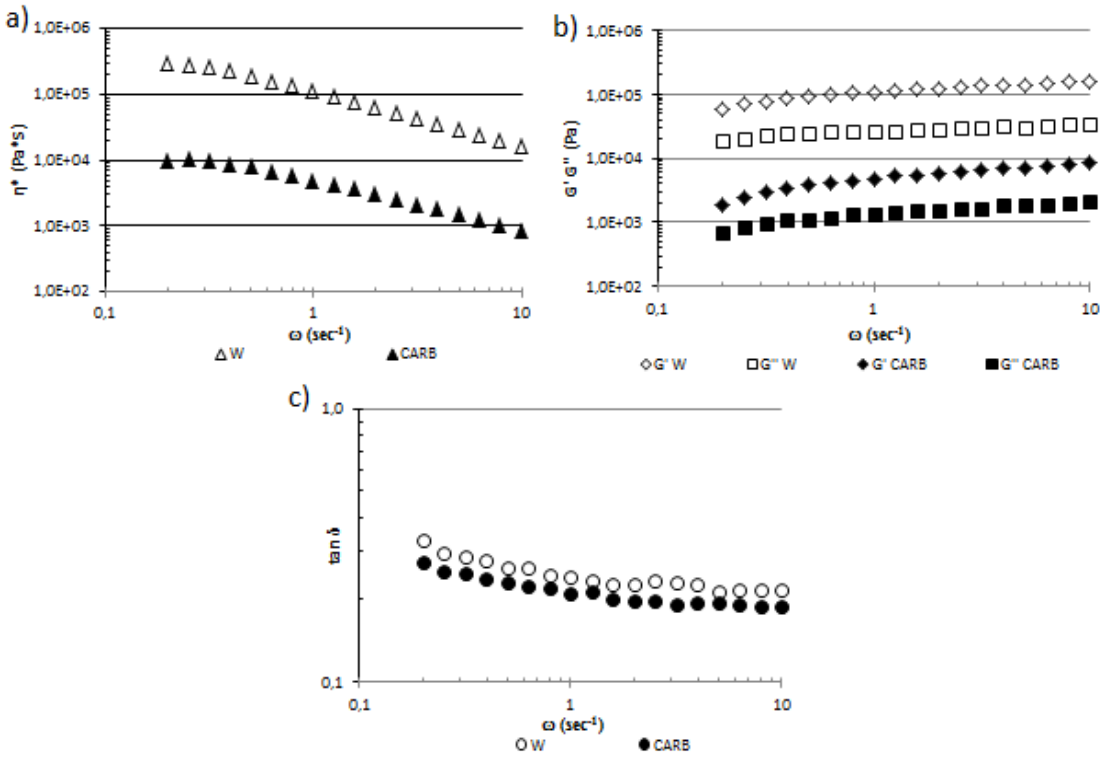


Figure 32: Rheological parameters of *in vitro* treated (full symbols) and untreated (empty symbols) mucus samples versus oscillation frequency (ω); a) complex viscosity b) elastic and viscous modulus c) Tan δ.

The preliminary results showed that the addition of sodium bicarbonate to the mucus sample caused a reduction in complex viscosity (Fig. 32a) with a

downward shift of both the elastic (rhombi) and viscous moduli (squares), The ratio of G'' to G' remains unchanged, as indicating by tan δ profiles almost overlapped (Fig. 32c). These results although encouraging need to be confirmed by further studies because of the paucity of the biological sample which prevents to perform all the required experiments.

2B 2.3 Permeation of ketoprofen lysine salt/leu from DPIs through CF native mucus and effect of $NaHCO_3$ on drug permeation

Permeation experiments were conducted on five mucus specimens (#1, #2, #3, #4, #5), obtained from different CF patients or different expectoration and using the Franz cells in a modified configuration interposing the mucus between the nitrocellulose membrane and the drug formulation (§4B 5).
As expected, the mucus layer slowed down drug dissolution and permeation compared to buffer permeability, acting as a physical barrier (Fig. 33).

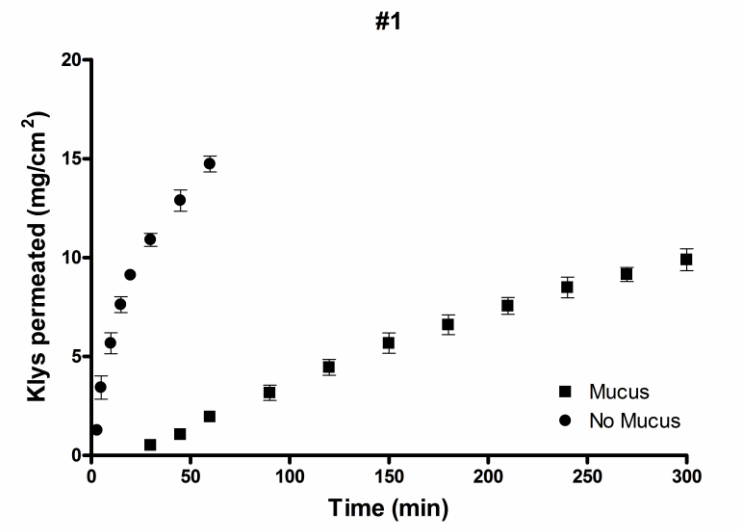


Figure 33: Mucus permeation profiles of ketoprofen lysinate (squares) in comparison with drug permeation profile without mucus (circles)

Afterwards, in order to evaluate the fluidizing effect of $NaHCO_3$ on the mucus permeation of Klys from the DPI, different experiments were performed with

and without the mucus layer. To rule out that $NaHCO_3$ effect on mucus was due to an osmotic effect, we decided to evaluate also the effect of NaCl solution isosmotic with $NaHCO_3$. Thus, it has been necessary to evaluate, firstly, the influence of water, $NaHCO_3$ and NaCl solutions and their respective pH on dissolution/permeation processes of Klys before studying the permeation through CF mucus.

To this purpose three experiments were performed dusting the powder onto the nitrocellulose membrane and adding 50 µl of each solution in the donor compartment. Figure 34 showed no significant difference in drug dissolution between the experiments carried out with water or with the salt solutions , indicating no salt effect on drug dissolution.

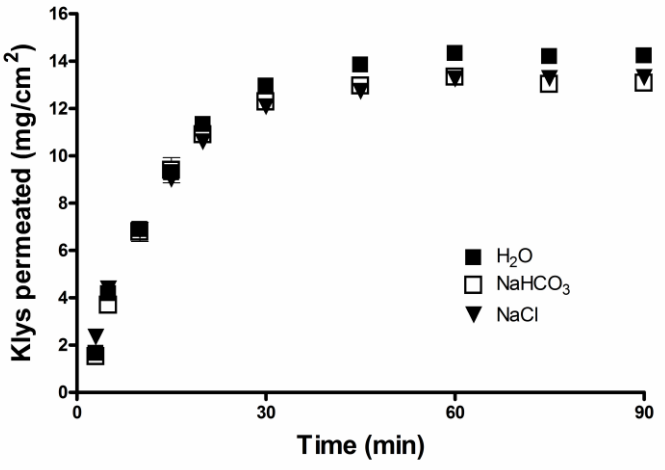


Figure 34: Permeation profiles of Klys obtained by adding 50 µl of H_2O (full squares), $NaHCO_3$ (empty squares) and NaCl (full triangles) in the donor compartment.

Moreover, permeation studies were repeated across the CF mucus treated *in vitro* with 50 µl of $NaHCO_3$ 0.5 M before starting the experiments.

The results in Figure 35 (#1-3) showed higher Klys permeation across CF mucus treated with $NaHCO_3$ compare to the untreated one, confirming the effect of $NaHCO_3$ in the reduction of mucus viscosity. On the contrary, no differences in drug permeation between the treated and untreated mucus were

observed for the sample #4. Probably, $NaHCO_3$ fluidizing effect is more evident when the mucus is more tenacious.

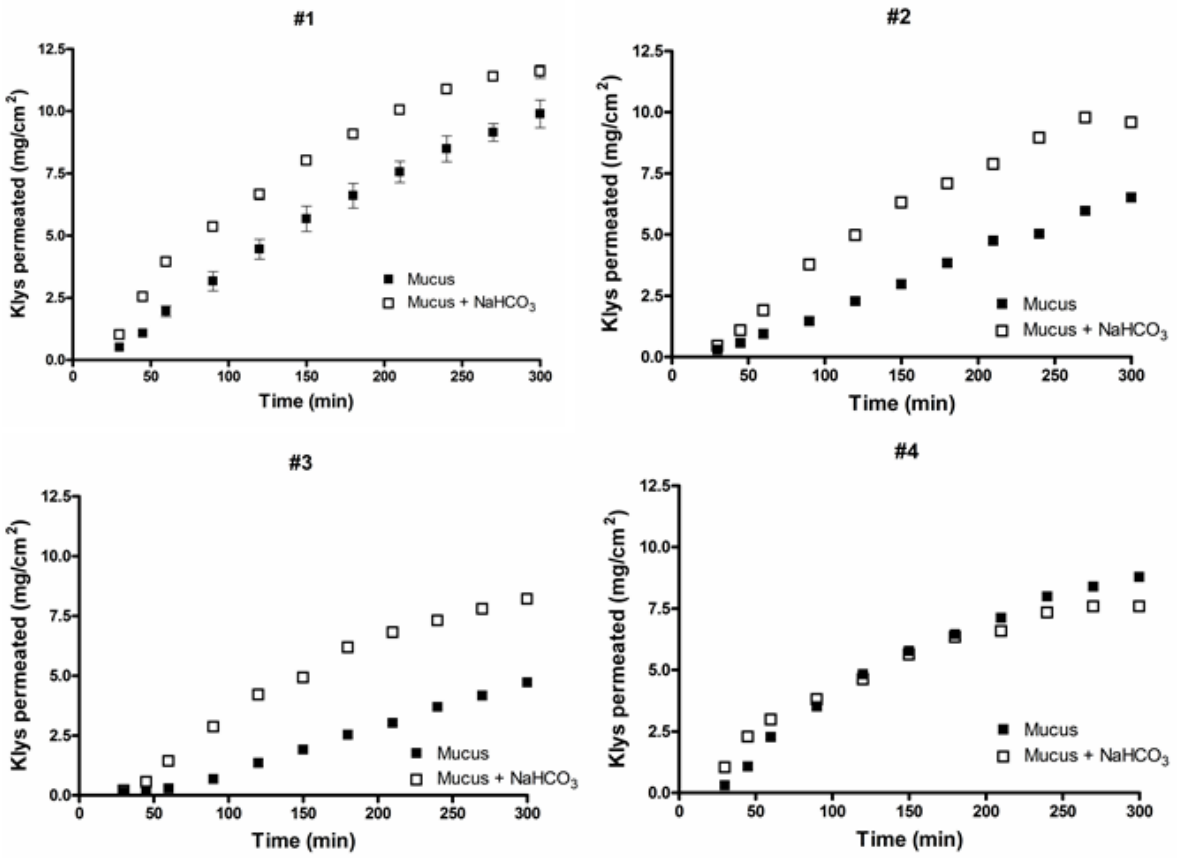


Figure 35: Permeation profiles of Klys through untreated (full squares) and treated (empty squares) mucus specimens (#1, #2, #3, #4).

Furthermore, to be sure that $NaHCO_3$ effect on mucus was not due to an osmotic effect, one more permeation test was performed treating the mucus with 50 µl NaCl solution isosmotic with $NaHCO_3$.

The results reported in Figure 36 showed that the permeation profiles obtained through the mucus treated with NaCl and the untreated mucus are completely overlapped confirming that $NaHCO_3$ acts reducing mucus viscosity.

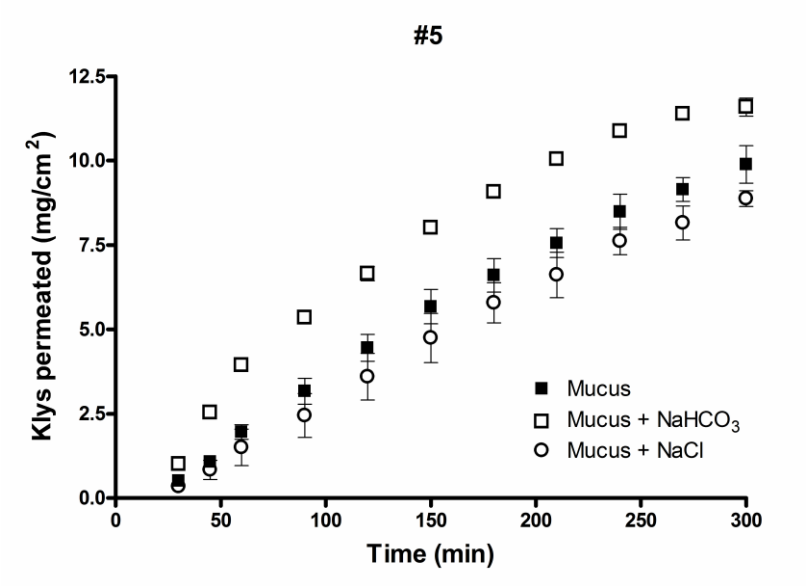


Figure 36: Permeation profiles of Klys through untreated mucus (full squares), mucus treated with $NaHCO_3$ (empty squares) and mucus treated with NaCl (empty circles).

3B 2.3 Conclusions

The preliminary but interesting results seem to confirm the role of $NaHCO_3$ in the swelling of the mucus. This weak base appears to act decreasing high viscosity of the CF bronchial secretion and, potentially, resulting in a better mucus clearance and in a barrier less difficult to overcame by inhaled drugs.
The valuable information obtained from these studies could suggest a possible use of $NaHCO_3$ as a therapeutic agent in the management of Cystic Fibrosis and, moreover, could be exploited to improve and develop a mucus model with chemical and rheological properties comparable with the CF mucus, allowing to perform all the required experiments.

SECTION C

EVALUATION OF ANTIBIOTIC TRANSEPITHELIAL TRANSPORT ACROSS CALU-3 HUMAN AIRWAY CELLS

Based on the articles: **Mariateresa Stigliani**, Mehra Haghi, Paola Russo, Paul M. Young and Daniela Traini "E*valuation of antibiotic transepithelial transport across calu-3 human airway cells", Respiratory Drug Delivery,* Puerto Rico **2014**. Vol 3, pp 703 – 706.

The final part of the project was performed at the Woolcock Institute of Medical Research in Sydney, under the supervision of Professors Daniela Traini and Paul M. Young. The research was aimed to study permeation processes of several antibiotics across Calu-3 cell line to obtain key information for the future formulation of inhaled products.

3C 1 Background, rationale and aim

Within the past few decades, the lung has received increasing attention as an effective pathway of targeted drug delivery for the treatment of chronic respiratory diseases and as an alternative route of systemic drug administration. Respiratory diseases such as tuberculosis, bronchiectasis, pneumonia, cystic fibrosis and chronic obstructive pulmonary disease benefit from direct administration of drugs to the lung in form of micronized droplets or solid microparticles. Indeed for some of these pathologies, inhaled antibiotic therapy has been developed to deliver the drugs directly to the site of action, reducing the dosing requirements, systemic toxicity and side effects (Geller 2009, Traini *et al.* 2009, Haghi *et al.* 2014). During the development of inhaled formulations it is crucial to understand the fate of the drug candidates after lung deposition, by considering the solubility of the drugs that could affect bioavailability. The Biopharmaceutics Classification System (BCS) established by Amidon and co-workers (Amidon *et al.* 1995), allows predicting the *in vivo* pharmacokinetics of the drugs but it is limited to the gastrointestinal absorption. Pulmonary administration requires an *ad hoc* study, taking into account the specific biology of the lung (metabolism, clearance, mucus and surfactant), as well as the characteristics of the formulation (Amidon *et al.* 1995, Eixarch *et al.* 2010, Cho *et al.* 2012).

Several methods have been employed to investigate pulmonary drug absorption such as *in vitro* cell culture methods, isolated lung perfusion

models and *in vivo* pharmacokinetic analyses (Mobley *et al.* 2001). Among these, *in vitro* cell cultures may be used as primarily screening tools to study the mechanisms involved in the absorption, metabolism and retention time of the drug in the airway epithelium (Borchardt 1995). A range of *in vitro* cell culture models of the respiratory epithelia have been employed for the screening of drugs candidate and moreover to study the pathological processes involved in lung diseases (Haghi *et al.* 2014).

Several studies have shown that Calu-3, an adenocarcinoma cell line derived from human bronchial epithelium, forms tight monolayers *in vitro*, produces secretory components and expresses several efflux and influx transporters (Mathia *et al.* 2002, Florea *et al.* 2003, Brillault *et al.* 2009), suggesting the usefulness of this cell line as an *in vitro* model to study pulmonary absorption.

The objective of this study was to investigate the correlation between physicochemical properties of different antibiotics, such as molecular weight, solubility, LogP and calculated permeability and their transport across Calu-3 cell line. The valuable information obtained from these studies, such as the rate of antibiotics transported across the epithelial cells and the impact of antibiotics physicochemical properties on their transport across the respiratory epithelia would be useful for the future formulation of inhaled antibiotics.

3C 2 Calu-3 viability

Firstly, the dose response cytotoxicity profile of antibiotics on Calu-3 cells was established. Calu-3 cell cultures were exposed to a range of antibiotics concentrations (from a minimum of 0.1 nM to a maximum of 250,000 nM) over a 72 h treatment period. Cells viability was calculated with reference to the untreated cells, where average absorbance was normalised to 100% viability. The viability results (Fig. 37), demonstrated that Calu-3 cells could tolerate different concentrations of the antibiotics used in the transport

experiments. With regards to the CPF toxicity, data have been previously reported in a study by Ong *et al.*, demonstrating that this antibiotic is not toxic at the concentrations used in our transport experiments (Ong *et al.* 2013).

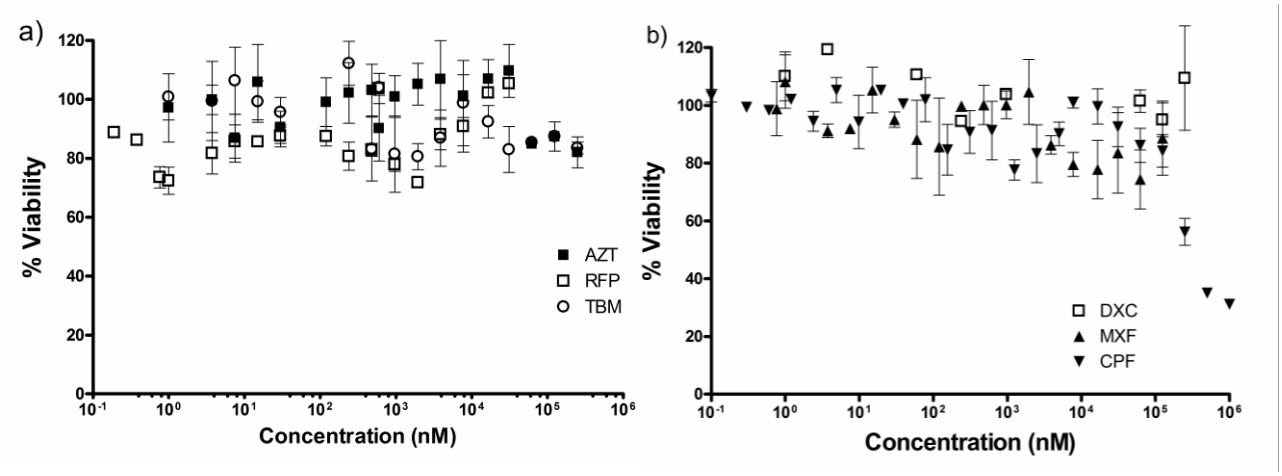


Figure 37: The effect of different concentrations of AZT, RFP, TBM (a) and DXC, MXF, CPF (b) on Calu-3 cell viability after 72 h drug treatment (n = 3, mean ± StDev).

3C 3 Calu-3 transport studies

The transport across Calu-3 cells was measured in both apical to basolateral (A-B) and basolateral to apical (B-A) directions as described in the experimental procedures (§4C 4).

For each antibiotic, the donor concentrations were selected based on the results obtained from cell viability, taking into account the solubility limit of each drug and the limit of detection by HPLC. The following are the concentrations of each antibiotic in the donor compartment: 10 and 20 µM for CPF, RFP and MXF, 20 and 40 µM for DXC, 100 µM for AZT and TBM, respectively.

Since the donor concentration (C_0) of the antibiotics were different, to facilitate comparison in terms of the amount transported and retained in the cells, the values were plotted as percentages. The percentage of antibiotics

transported after 180 minutes in the absorptive (A-B) and secretory directions (B-A) is shown in Figure 38a and b.

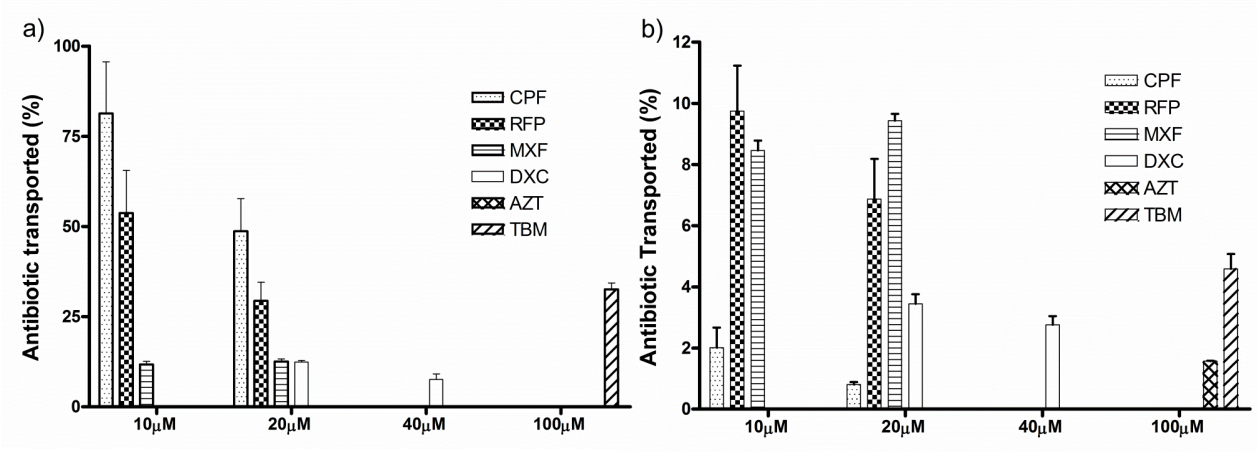


Figure 38: Percentage of CPF, RFP, MXF, DXC, AZT, TBM transported from A-B (a) and B-A (b) direction across Calu-3 cells after 180 minutes (n = 3, mean ± StDev).

Apical to basolateral transport of CPF was assayed at 10 and 20 µM. Results showed that approximately 81.00 ± 24.74 % of 10 µM CPF and only 48.65 ± 15.70 % of the 20 µM CPF was transported across Calu-3 cells. This finding indicates that the transport of CPF was independent of the initial concentration and was not transported exclusively through the paracellular route, indicating that influx transporters could be involved in the transport of CPF. B-A transport showed that only a small percentage of CPF (2.01 ± 1.13 % at 10 µM and 0.80 ± 0.14 % at 20 µM) was transported in the secretory direction. These results are in good agreement with findings from Ong *et al.* where it was shown that when specific active transporter inhibitors are present, the permeability of CPF in both A-B and B-A directions was decreased (Ong *et al.* 2013).

Figure 38a showed that 53.70 ± 20.50 % of 10 µM RFP and only 29.40 ± 8.90 % of 20 µM RFP was transported in the absorptive direction. These results suggested that active transporters can play a role in RFP absorption. Currently, there are only few studies on the transport mechanisms for rifampicin in

organs such as intestine and lungs. Tewes *et al.* and Mariappan *et al.* found that RFP permeation across Calu-3 cells and intestinal segments is driven by a saturable mechanism (Mariappan *et al.* 2004, Tewes *et al.* 2008). Hence, as the drug concentration rises, its transport increases up to the point where all the transport carriers involved become saturated.

MXF absorption and secretory transport experiments revealed that the percentage of MXF transported was low both in A-B direction (11.80 ± 1.40 % for C_0 of 10 µM) and in B-A direction (12.60 ± 1.55 % for C_0 of 20 µM and 8.50 ± 0.56 % for C_0 of 10 µM and 9.43 ± 0.38 % for C_0 of 20 µM). Moreover no significant difference ($P>0.05$) in the percentage of antibiotic transported was found between the two different concentrations studied, indicating a possible passive or paracellular absorption of the antibiotic depending on the concentration gradient of the drug in the donor and receiver chambers. These results are in agreement with the study by Brillault *et al*., that reported MXF to be a substrate of P-glycoprotein, a well-known membrane efflux transporter overexpressed in the apical and basolateral sides of Calu-3 cells. The presence of this efflux transporter prevents the MXF to remain inside the cells (Brillault *et al.* 2009), further confirmed by our results in Figure 39 showing the percentage of antibiotics recovered inside the cells.

Similarly, DXC showed a low absorptive and secretory permeation (12.43 ± 3.65 % for C_0 of 20 µM and 7.60 ± 2.60 % for C_0 of 40 µM in A-B direction and 3.44 ± 0.53 % for C_0 of 20 µM and 2.75 ± 0.48 % for C_0 of 40 µM in B-A direction) probably due to efflux pumps. Moreover, as shown in the Figure 39, the percentages of MXF and DXC recovered inside the cells were less than 1.00 %, attesting that these antibiotics are unable to enter or remain in the cells.

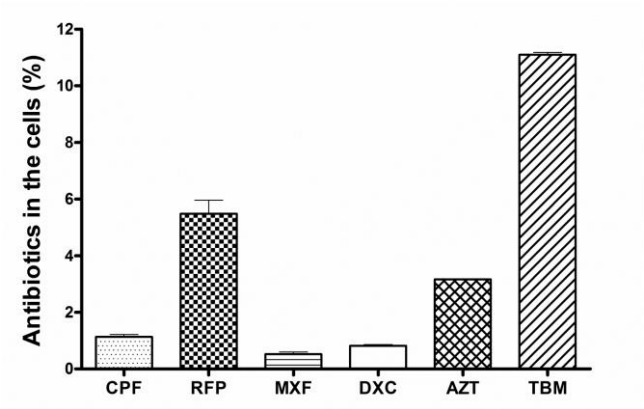


Figure 39: Percentage of CPF, RFP, MXF, DXC, AZT, TBM recovered inside the cells after 180 minutes (n = 3, mean ± StDev).

As reported in Figure 38a AZT did not show A-B transport. The analysis of epithelial cells drug content revealed that after 3 hours only a small percentage of drug (3.16 ± 0.03 % of 100 µM) was found inside the cells (Fig. 39), and 90.00 ± 0.60 % of AZT 100 µM was found remaining on the cells. Small percentage of AZT (1.57 ± 0.02 % of 100 µM) was transported in B-A direction. These results were in agreement with a study by Pachot *et al.*, in which the transport of different macrolides was examined across Caco-2 cell monolayers and demonstrated the involvement of P-glycoprotein as efflux pump in the both absorptive and secretory directions (Pachot *et al.* 2003).
Finally, TBM was assayed at different concentrations (ranging from 20 to 100 µM) but it was only detected in the basolateral compartment (A-B direction), when 100 µM initial concentration was used, showing TBM to have low permeability. Further studies are required to investigate whether the observed results are due to the involvement of the efflux transporters.

3C 4 Effect of the molecular weight (MW) and logP on the P_{app}

The epithelial permeability of these antibiotics was calculated as reported in the experimental procedures (§4C 5) and investigated in relation to their physicochemical characteristics, presented in Table 9.

Table 9. Water solubility, partition coefficient (LogP) and molecular weight (MW) of the antibiotics.

Drug	Water Solubility	LogP	MW (g/mol)
CPF	sps*	1.32	367.84
MXF	s*	0.01	437.89
RFP	vss*	3.72	822.94
DXC	s*	-4.17	480.89
AZT	sps*	4.02	785.02
TBM	s*	-7.32	467.51

*Soluble (s), sparingly soluble (sps), very slightly soluble (vss).

Transport of the antibiotics across Calu-3 was calculated as the P_{app}, cm/s (§ 4C 5). Calculation of P_{app} for AZT (C_0 20 µM) was not possible in the absorptive direction, since no drug was detected in the aliquot sampled from the basolateral compartment. To confirm that this antibiotic did not permeate/transport through the Calu-3 monolayer, the experiment was repeated using higher concentration of AZT (ranging from 20 up to 100 µM) and sampling only after 180 min; however, even in this case, no drug was detected in the receiver compartment.

With regard to TBM, different concentrations (ranging from 20 to 100 µM) were tested, however, only at 100 µM and following sampling after 180 minutes, 32.60 ± 3.03 % of initial TBM concentration, was detected in the

basolateral compartment; consequently, even in this case, the lack of enough drug concentration points transported over time prevented the calculation P_{app}. In Figure 40a, the P_{app} values of the different antibiotics transported from apical to basolateral direction (C_0 20 µM) are plotted as a function of their molecular weights. The P_{app} of RFP ($6.40 \times 10^{-4} \pm 1.06 \times 10^{-4}$ cm/s) was the highest in spite of its high molecular weight. CPF ($3.50 \times 10^{-4} \pm 1.53 \times 10^{-4}$ cm/s), DXC ($2.02 \times 10^{-4} \pm 2.82 \times 10^{-5}$ cm/s) and MXF ($1.59 \times 10^{-4} \pm 1.46 \times 10^{-5}$ cm/s), both with low and similar molecular weights, showed different values of permeability. These findings indicate that antibiotics transport is independent of their molecular size.

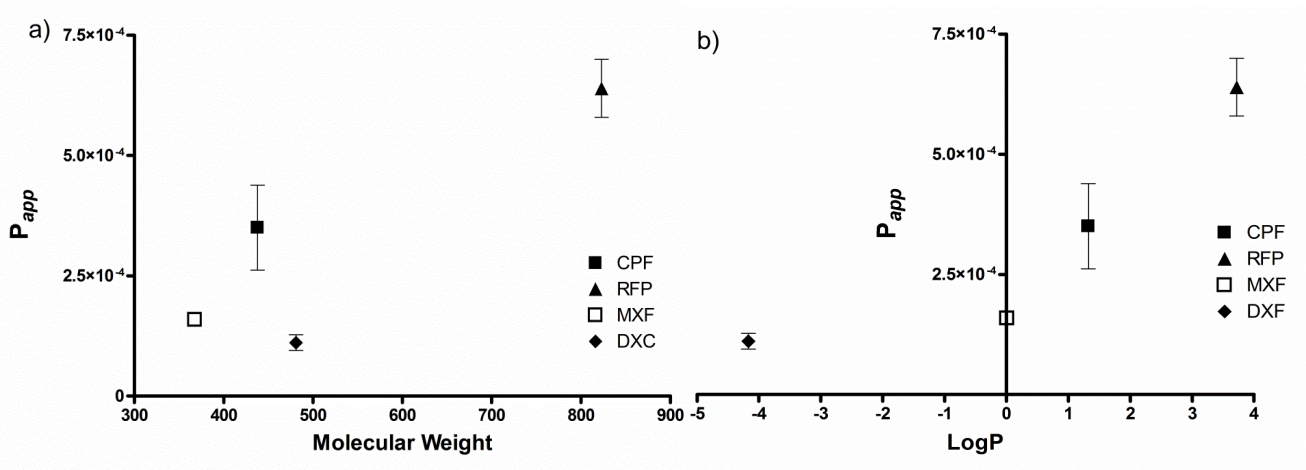


Figure 40. Apparent permeability (P_{app}) vs. Molecular weight (MW) (a), and LogP (b) from the apical to basolateral across Calu-3 (n=3, mean± StDev).

The relationship between the P_{app} and the partition coefficient (LogP) of the antibiotics in this study is shown in Figure 40b. Generally, the P_{app} (A-B direction) increased with an increase of LogP. This finding is in agreement with previous studies where drug lipophilicity has shown to increase the transport across the epithelium (Bur *et al.* 2010). Hence, the highest P_{app} of RFP and CPF are in agreement with the higher LogP values, compared to MXF and DXC the most hydrophilic of the investigated antibiotics,

accordingly to their lowest P_{app} values. Moreover, the P_{app} of RFP, a lipophilic drug, led to the hypothesis that passive transport mechanisms could occur in addition to the active and saturable transport, as previously discussed.

Basolateral to apical studies (Fig. 41a) showed that RFP ($3.58 \times 10^{-4} \pm 1.27 \times 10^{-4}$ cm/s) had the highest P_{app} value, followed by MXF ($2.76 \times 10^{-4} \pm 1.02 \times 10^{-5}$ cm/s), DXC ($1.31 \times 10^{-4} \pm 2.50 \times 10^{-5}$ cm/s) and CPF ($1.99 \times 10^{-5} \pm 3.32 \times 10^{-6}$ cm/s), respectively, indicating no relationship between the P_{app} and the molecular weight of the antibiotics. Furthermore, for most antibiotics in this study (DXC, MXF and RFP), P_{app} increased with an increase of LogP, except for ciprofloxacin (average LogP of 1.32) that showed the lowest permeability ($1.99 \times 10^{-5} \pm 3.32 \times 10^{-6}$ cm/s) (Fig. 41b).

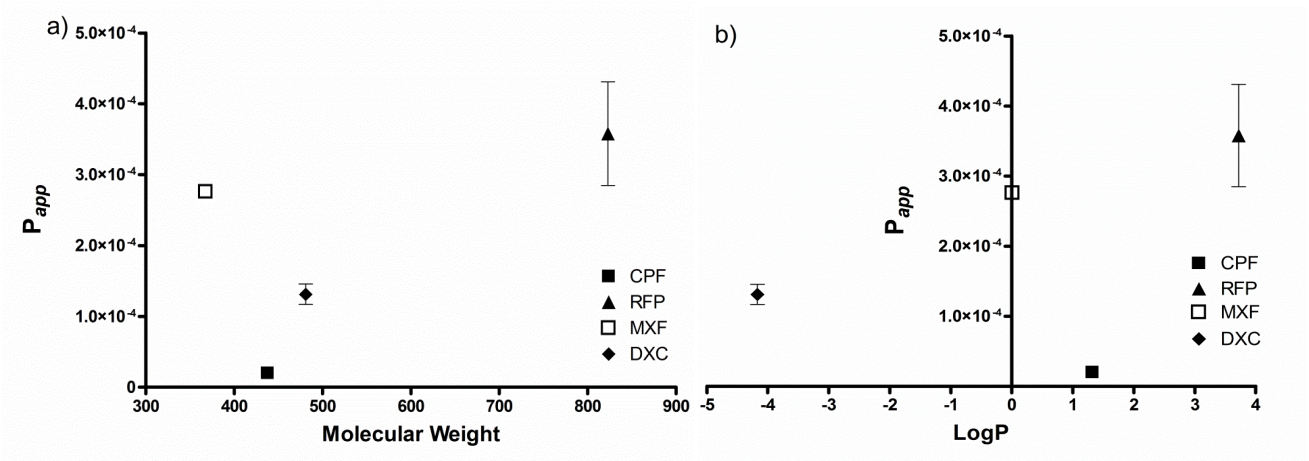


Figure 41. Apparent permeability (P_{app}) vs. Molecular weight (MW) (a), and LogP (b), from the basolateral to apical direction across Calu-3 (n=3, mean± StDev).

On the basis of these results, generally all antibiotics studied (except for MXF) showed lower permeabilities in the secretory direction compared with the absorptive direction. These findings suggest that the inhalation route would be favourable for delivering these antibiotics for the treatment of respiratory diseases where infection is present, compared with the present oral or

intravenous administration that requires high doses of antibiotic to ensure its sufficient concentration in the lungs (Ong *et al.* 2013).

3C 5 Conclusions

In this study the *in vitro* relationship between physicochemical properties of antibiotics for inhalation therapies and their P_{app} values across human bronchial epithelial cell monolayer was investigated. Inverse relationship between MW of the antibiotics and permeability was shown both in the absorptive and secretory directions. A direct relationship was shown between antibiotics permeability and LogP in apical to basolateral transport, while the opposite was found for the basolateral to apical.

These results provide information critically important for the future formulation of antibiotics for pulmonary delivery, for both local and systemic applications.

EXPERIMENTAL PROCEDURES

SECTION A (Parts 1 and 2)
DESIGN AND DEVELOPMENT OF DPIs CONTAINING KETOPROFEN LYSINE SALT TO TREAT INTRINSIC INFLAMMATION IN CF PATIENTS

4A 1 Chemicals

Ketoprofen and ketoprofen lysine salt were kindly donated by Dompè spa (L'Aquila, Italy); L-leucine, was supplied by Sigma Aldrich (Milan, Italy). Isopropyl alcohol (for analysis, USP grade) was purchased from Carlo Erba Reagents (Milan, Italy). Other solvents and chemicals were of analytical grade. Size 3 gelatine capsules were purchased from Dermolife (Trento, Italy). The device used for aerodynamic tests was the monodose DPI model 7 kindly donated by Plastiape SpA (Milan, Italy). All the cell culture reagents were purchased from Lonza (Milan, Italy).

4A 2 Powders preparation *via* Mini Spray Drying

Micronized particles, containing ketoprofen or ketoprofen in its lysine salt form, were prepared by spray drying the drug alone or with leucine (85:15 w/w, respectively) as dispersibility enhancer from water or water/isopropyl alcohol 7/3 v/v mixtures. Drug alone or with leucine was solubilized in water, then the organic solvent was added under continuous magnetic stirring. The total powder concentration reached 5% w/v in all batches.

The liquid feeds containing ketoprofen (pH=3.3) were neutralized with few drops of a 1 M sodium hydroxide solution. All the solutions were dried using a Buchi mini spray dryer B-191 (Fig. 42) (Buchi Laboratoriums-Tecnik, Flawil, Switzerland) under the following operative conditions: inlet temperature 110 °C, outlet temperature 72-75°C, drying air flow 500 L/min, aspiration rate 100%, air pressure 6 atmospheres, feed rate 5 ml/min, nozzle 0.5 mm, set in preliminary experiments.

Figure 42. BÜCHI B-191 Mini Spray Dryer.

Each preparation was carried out in triplicate and all the spray-dried powders were collected and stored under vacuum for 48 h at room temperature. Production yields were expressed as weight percentage of the final product over the total amount of sprayed material.

4A. 1.3 Powders preparation *via* Nano Spray Drying

Micronized particles were prepared using BÜCHI B-90 Nano Spray Buchi (Fig. 43) (Laboratoriums-Tecnik, Flawil, Switzerland) from different hydro-alcoholic solutions (IPA from 0 to 30% v/v) containing ketoprofen in its lysinate salt form and leucine as dispersibility enhancer in different ratios (from 5 to 15% w/w) with a total solid concentration ranging from 1 to 7% w/v. Besides solutions compositions, the operating conditions of nano spray drier were tuned in order to study their effect on powder technological properties.

Figure 43: BÜCHI B-90 Nano Spray

In details, inlet temperature ranged between 60 and 110°C, while air flow rate (100 L/min), feed rate (1.5 ml/min), and relative spray rate (100%) were kept constant. Solution were sprayed alternatively using nozzles with mesh diameters of 4.0, 5.5 and 7.0 µm. A nozzle pre-treatment with surfactant solutions was also carried out, aiming to reduce the processing time.

4A. 1.4 Powders physico-chemical characterization

4A. 1.4a Ketoprofen lysinate quantification

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

4A. 1.4b Particle size

Particle size of both raw materials and engineered particles was determined using a light-scattering laser granulometer equipped with a tornado powder

dispersing system (LS 13 320 Beckman Coulter Inc., FL, USA). The LS 13 320 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. The particle size was obtained by a specific software using Mie theory to produce an optimal analysis of the light energy distribution. The tornado module leads to a dispersion similar to the one achieved when the samples are run wet, without using any solvent which can alter powder surface properties (Stewart *et al.* 2009). 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, defined as $[d_{(90)} - d_{(10)}]/d_{(50)}$, where $d_{(10)}$, $d_{(50)}$ and $d_{(90)}$ indicate diameters at the 10th, 50th and 90th percentiles of the particle size distribution, respectively.

4A. 1.4c Particle morphology

Morphology of raw materials and microparticles was examined using a scanning electron microscope (SEM) Zeiss EVO MA10 (Carl Zeiss SMT AG, München-Hallbergmoos, Germany) operating at 14 kV.

4A. 1.4d Differential scanning calorimetry analysis

Differential scanning calorimetry (DSC) was performed with an Indium-calibrated Mettler Toledo DSC 822e (Fig. 44) (Mettler Toledo, OH, USA).

Figure 44: DSC 822[e].

Accurately weighed samples (3-5 mg) (MTS Mettler Toledo microbalance, OH, USA) were placed in a 40 μL aluminium pan, which was sealed, pierced and heated up to 350°C at a heating rate of 20°C/min.

4A. 1.4e Bulk and tapped density

Bulk density of the spray-dried powders was measured as described elsewhere (Sansone *et al.* 2009). Briefly, powders were loaded into a bottom-sealed 1 ml plastic syringe (Terumo Europe, Leuven, Belgium) capped with laboratory film (Parafilm® "M", Pechiney Plastic Packaging, Chicago, IL, USA) and tapped on a hard bench until no change in the volume of the powder was observed. The bulk and tapped densities were calculated from the net weight of the plastic syringe content divided by the powder volume in the syringe before and after tapping, respectively. Experiments were performed in triplicate.

4A. 1.5 Aerodynamic behavior evaluation

Powders aerodynamic properties were assessed by Andersen cascade impactor (apparatus D, Eur. Ph. 6.0, ACI, Westech Instrument Services Ltd., Bedfordshire, UK) and the effective cut-off diameters of the ACI (Fig. 45) were: Stage −1, 8.6 μm; Stage −0, 6.5 μm; Stage 1, 4.4 μm; Stage 2, 3.2 μm; Stage 3, 2.0 μm; Stage 4, 1.1 μm; Stage 5, 0.54 μm; Stage 6, 0.25 μm modified for use at a flow rate of 60 L/min as described elsewhere (Sansone *et al.* 2009).

Figure 45: Andersen cascade impactor.

The device used to aerosolize the powders was the *monodose*® (Plastiape, Milan, Italy) (Fig. 46), a breath-activated, reusable DPI, working with a size 3 capsule.

Figure 46: *Monodose*® DPI

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 empty. In order to minimize particle bounce, metal impaction plates were dipped into an *n*-hexane solution of SPAN 80 (0.1% w/v) and the solvent was allowed to evaporate, leaving a thin film of SPAN 80 on the plate surface. The ACI was assembled placing a filter paper on the filter stage and the *monodose* DPI was fitted into a rubber mouth piece attached to the metal throat. Four hard gelatine

capsules (size 3) were filled manually with 40 ± 0.5 mg of sample. Each capsule was introduced into the *monodose* DPI and pierced. The vacuum pump was actuated for 4 s. The powder deposited into the different stages was recovered by plunging each plate and the stage below into distilled water (5-500 ml depending on the stage number). Drug content was assessed by UV measurements. The emitted dose (ED) was gravimetrically determined and expressed as percentage of powder exiting the device versus the amount of powder introduced into the capsule. The cumulative mass of powder with a diameter lower than the stated size of each stage was calculated and plotted as a percentage of recovered powder *vs* cut-off diameter. The mass median aerodynamic diameter of the particles was extrapolated from the graph, according to the Eur. Ph. 6.0. From the same plot, the fine particle dose (FPD), i.e. the mass of drug with a particle size less than 5 μm, and the fine particle fraction (FPF), i.e. the fraction of drug emitted from the device with a particle size less than 5 μm, were determined. *In vitro* deposition experiments were performed on three batches with three replicates each.

4A. 1.6 Biological activity

4A. 1.6a Cell lines and culture conditions

CuFi1 cell line, derived from human bronchial epithelium of a CF (CuFi1, CFTR ΔF508/ΔF508 mutant genotype) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). CuFi1 cells were grown in human placental collagen type VI coated flasks (Sigma–Aldrich, Milan, Italy) in bronchial epithelial basal medium BEGM (Clonetic Lonza Walkersville. Inc) supplemented with BPE, hydrocortisone, hEGF, epinephrine, insulin, triiodothyronine, transferrine and retinoic acid (all from Lonza) and

penicillin/streptomycin (50 mg/ml). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

For *in vitro* biological studies, stock solutions of powders were prepared in sterile water, and then opportunely diluted with BEBM and immediately administered to the cells.

4A. 1.6b Proliferation assay

Cell growth was assessed by using a colorimetric bromodeoxyuridine (BrdU) cell proliferation ELISA kit (Roche Diagnostics, Milan, Italy). Briefly, 5×10^3 cells were seeded into each coated well of a 96-well plate and left to adhere to the plate. The cells were then treated with increasing concentrations (from 0.1 to 1000 µM, expressed as ketoprofen content) of raw ketoprofen, raw ketoprofen lysine salt, Klys-1a and Klys-2a for 48 h. BrdU was added for the final 16 h (10 µM final concentration). At the end of the whole cell culture period, the medium was removed and the ELISA BrdU immunoassay was performed as described by the manufacturer. The colorimetric reaction was stopped by adding H_2SO_4, and the absorbance at 450 nm was measured using a microplate reader (Bio-Rad Laboratories, Milan, Italy).

4A. 1.7 Statistical analysis

Measurements were performed in triplicate, unless otherwise stated. Values were expressed as means of at least three experiments with three replicates each ± SD. Statistical differences between the treatments and the control were evaluated by the Student's *t*-test A (*P* values less than 0.05 were considered statistically significant).

SECTION B (Part 1 and 2)
DRUG PERMEATION STUDIES THROUGH ARTIFICIAL CF MUCUS AND HUMAN BRONCHIAL SECRETIONS

4B 1 Chemicals

Ketoprofen lysine salt was kindly donated by Dompè spa (L'Aquila, Italy); Gentamicin sulphate (G), L-leucine (leu), o-phthalaldehyde, Isopropanol (for analysis, USP grade) and sodium hydroxide anhydrous pellets used for the G quantification were supplied by Sigma Aldrich (Milan, Italy). Other solvents and chemicals were of analytical grade. Deoxyribonucleic acid, mucin from porcine stomach, diethylenetriaminepentaacetic acid (DTPA), RPMI 1640 Amino Acids Solution, egg yolk emulsion, sodium chloride and potassium chloride used for artificial mucus preparation were supplied by Sigma Aldrich (Milan, Italy) and high viscosity hydroxyethylcellulose by A.C.E.F. SpA (Piacenza, Italy).

4B 2 CF artificial mucus

Basic CF artificial mucus (AM-) was firstly prepared as previously reported (Sriramulu *et al.* 2005). Briefly, 4 g of deoxyribonucleic acid (DNA), 5 g of mucin, 5.9 mg of diethylenetriaminepentaacetic acid (DTPA), 20.0 ml of RPMI 1640 Amino Acids Solution, 5 ml of egg yolk emulsion, 5.0 g NaCl and 2.2 g of KCl were mixed together in 1l of distilled water (final volume). The pH of the resulting solution was adjusted to 7.0 ± 0.1 by a NaOH solution. However, AM^- was too fluid (viscosity not detected) and passed through the membrane pores during 60 min, preventing the permeation study. Consequently, two more different models, AM_1 and $AM_{1.5}$, were prepared, adding 1% and 1.5% w/v of HEC (hydroxyethylcellulose), respectively, as an inert thickening agent to the previous formula. These solutions were allowed to equilibrate at 25°C for 12h before any analysis or use, and characterized for their rheological properties.

4B 3 Viscosity measurement of artificial mucus

Viscosity of AM_1 and $AM_{1.5,}$ was determined by means of a rotational viscometer (Smart Series, Fungilab SA, Barcelona, Spain), based on the measurements of the torque of a rotating spindle in a sample at a specified velocity. Samples were inserted in a 150 ml glass becker. Viscosity tests were conducted using the R2 spindle at different velocity (100-60-50-30-20-10 rpm). Data were reported as Pa×S related to the rotor velocity (ω); the resulting viscosity values were always between 15% and 100% of the torque range, as requested.

4B 4 Rheological measurement of CF mucus patients

Rheological measurements were performed using an ARES rotational rheometer (Rheometrics, Inc.) with a parallel plates geometry (plate diameter 25 mm, gap of 0.5 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. Four parameters were identified dependent on frequency (ω, s^{-1}): η* (complex viscosity), G' (elastic modulus), G'' (viscous modulus) and tan δ (ratio of G'' to G'). All samples were tested at 37°C and all experiments were carried out under air flux.

4B 5 *In vitro* permeation study

Permeation assays were performed by means of Franz-type vertical diffusion cells (Hanson research corporation, CA, USA).

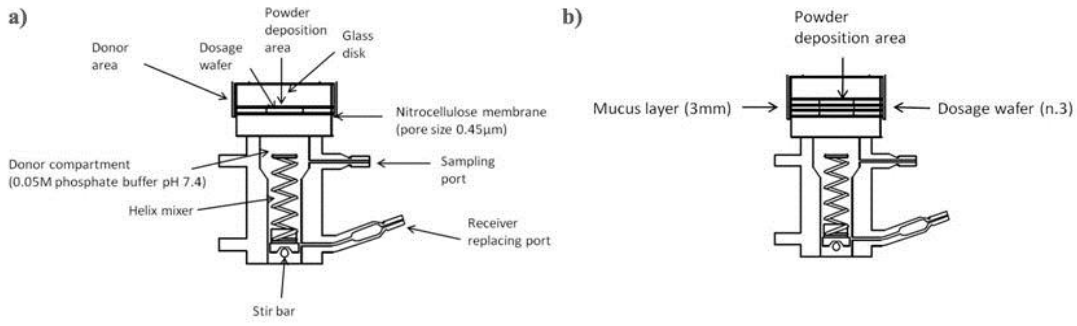


Figure: 47. Franz cell used in permeation studies: a) standard configuration with one dosage wafer housing the drug formulation; b) modified configuration with three dosage wafers housing mucus and drug formulation.

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. Firstly, permeation experiments were conducted with Franz cells in a standard configuration (Fig. 47a), spreading the powder directly on the membrane. The receptor compartment was filled with 7 mL of a 0.05 M phosphate buffer (pH 7.4) and a nitrocellulose membrane (size pores: 0.45µm), previously set with phosphate buffer, was applied between the two compartments (permeation area 1.77 cm^2). About 35 mg of the selected powder, precisely weighed, were uniformly dusted on the membrane surface in the dosage wafer, which was subsequently sealed with spring clips and laboratory film (Parafilm®). Samples (200 µl) were removed at defined time intervals (5, 10, 15, 20, 30, 45, 60 min) inserting the same volume of warmed buffer. 100 µl aliquots were analyzed for drug content. The amount of the drug permeated *per* area (Q) for each time interval was calculated by means of the following equation:

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

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).

Permeation data were reported as the quantity of permeated drug *per* permeation area (mg/cm^2) related to time.

In a second set of experiments, Franz cells were used in a modified configuration (Fig. 47b) (Donnelly *et al.* 2007): a thin layer (3mm) of artificial mucus AM_1 or $AM_{1.5}$ was interposed between the nitrocellulose 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, 60, 90, 120, 150, 180 min) and analyzed.

4B 6 Gentamicin Sulfate quantification

G quantitative determination by HPLC followed the Pharmacopoeia method (USP 30) as reported elsewhere (Della Porta *et al.*). Briefly, 25 mg of G raw material was stirred in 25 ml of distilled water until complete dissolution. Five ml of IPA and 4 ml of a previously prepared phthalaldehyde solution were then added to 10 ml of this solution. The solution was stirred and IPA was added to reach a 25 ml volume. Finally, it was heated for 15 min in a water bath at 60 °C, cooled at room temperature, filtered through 0.45 µm filters and analyzed by HPLC at a wavelength of 330 nm (Chromatopac L-10AD system equipped with a Model SPD-10AV UV–vis detector and a Rheodyne Model 7725 injector loop 20 µl, Shimadzu, Kyoto, Japan). Peak areas were calculated with a Shimadzu C-R6A integrator. Phthalaldehyde solution was obtained dissolving 1.0 mg of o-phthalaldehyde in 5 ml of methanol and adding 95 ml

of 0.4 M boric acid, previously adjusted with 8 N KOH to a pH of 10.4, and 2 ml of thioglycolic acid. The pH of the resulting solution was adjusted to 10.4 by a 8 N KOH solution. Calibration curves were worked out and proportionality between G concentration and AUC was checked in the range of 5-500 μg/ml.

After adding the phthalaldehyde solution to a sample containing both G and leu, the amino acid reacted with phthalaldehyde, giving rise to a chromophore absorbing at 330 nm, as observed for G, with no interference with G. Calibration curves were worked out for leu, too, and proportionality between leu concentration and AUC was tested in the range of 1-20 μg/ml.

4B 7 Ketoprofen lysinate quantification

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

SECTION C:
EVALUATION OF ANTIBIOTIC TRANSEPITHELIAL TRANSPORT ACROSS CALU-3 HUMAN AIRWAY CELLS

4C 1 Chemicals

Calu-3 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Nonessential amino acids solution, CelLytic M Cell Lysis, protease inhibitor cocktail and dimethyl sulfoxide (DMSO), were purchased from Sigma-Aldrich (Sydney, Australia). Other cell culture reagents including trypsin-EDTA solution (2.5 g/L trypsin, 0.5 g/L EDTA), Dulbecco's modified Eagle's medium (DMEM, without phenol red and L-glutamine, including sodium bicarbonate and 15 mM HEPES), Trypan blue solution (0.4 % w/v), phosphate buffered saline (PBS), L-glutamine solution (200 mM), fetal bovine serum (FBS), and Hanks balanced salt solution (HBSS) were obtained from Invitrogen (Gibco, Invitrogen, Sydney, Australia). Transwell cell culture inserts (0.33 cm^2 polyester, 0.4 μm pore size) were purchased from Corning Costar (Lowell, MA, USA), and all other sterile culture plastic wares were from Sarstedt (Adelaide, Australia). All solvents used were of analytical grade and were supplied by Biolab (Victoria, Australia). Ciprofloxacin (CPF) HCl, azithromycin (AZT) dihydrate and moxifloxacin (MXF) HCl were purchased from Sigma-Aldrich (Sydney, Australia), rifampicin (RFP) was purchased from Hangzhou ICH Imp & Exp Company Ltd. (Hangzhou, China), doxycycline (DXC) HCl was purchased from MP Biomedicals Inc (Ohio, USA) and tobramycin (TBM) was kindly gifted from Lisapharma S.P.A (Erba, Italy).

4C 2 Cell culture

The Calu-3 cell line (HTB-55) between passages 35 and 42 were maintained in tissue culture flasks of 75 cm^2 and cultured in pre-warmed DMEM supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) nonessential amino acid solution, and 1% (v/v) L-glutamine solution. Cells were incubated at

37°C in 5% CO_2 and 95% humidity until confluence was reached. Medium was exchanged every 2 to 3 days, and the cells were passaged weekly, according to ATCC-recommended guidelines. Calu-3 cells were grown at the air interface to allow monolayer differentiation. These conditions have previously been established by Haghi *et al*. (Haghi *et al.* 2010) and Grainger *et al*. (Grainger *et al.* 2006). Cells were seeded at a density of 5.0×10^5 cells/cm^2 in the apical compartment of the 24-well Transwell cell culture inserts in 100 µl medium and 500 µl medium was added to the basolateral chamber. Subsequently, the medium in the apical chamber was removed after 24 h and was replaced in the basolateral chambers only every 2 to 3 days. The transport experiments were performed between days 11 and 14 after the seeding.

4C 3 Viability of Calu-3 cells following exposure to antibiotics

The viability of Calu-3 cells following exposure to different concentrations of antibiotics were assessed by colorimetric MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2Htetrazolium) *in vitro* assay, according to the method described by Scalia *et al.* (Scalia *et al.* 2013). Briefly, 5×10^4 cells were seeded per well in a volume of 100 µl into a 96 well plate. Cells were incubated overnight at 37°C in 5% CO_2 atmosphere.

Concentrations of the antibiotics assessed were as following: CPF up to 50 uM, MXF from 8.0×10^{-4} to 100 µM, RFP from 1.0×10^{-4} to 25 µM, AZT, DXC, CPF and TBM from 1.0 to 250 µM. After 24 h, 100 µl of increasing concentrations of antibiotics were added to each well. The stock solution of each antibiotic was prepared by dissolving the drug in ethanol or DMSO and further diluting it with the complete medium to the final ethanol concentration of ⩽1%. Untreated and solvent controls were included for each experiment. Plates were incubated for 72 h at 37°C in a humidified atmosphere with 5% CO_2. Cells were then analysed for cell viability. Briefly, 20 µL of CellTiter

96® Aqueous assay (MTS reagent) (Promega, USA) was added to each well and the plates incubated for 3 h at 37°C in humidified 5% CO_2 atmosphere. Absorbance was measured at 492 nm using a plate reader (Spectramax M2 and Soft Max pro 4.8, Molecular Devices, Sunnyvale, CA, USA). The absorbance value was directly proportional to cell viability (%). Data were expressed as % cell viability [(average absorbance of treated wells/average absorbance of control wells) × 100]. The half maximal inhibitory concentration (IC_{50}) values were defined as the drug concentration that produces a decrease of 50% in cell viability compared to the untreated control. IC_{50} values were calculated by plotting (%) cell viability against the concentrations ($ng.ml^{-1}$) on a logarithmic scale. Data was fitted to the Hill equation using the General Fit function of KaleidaGraph 4.1 software. Each experiment was performed in triplicate.

4C 4 Calu-3 transport experiments of the antibiotics

The transport across the cells was measured in both apical to basolateral (A-B) and basolateral to apical (B-A) directions. Schematic diagram of transport experiments is reported in Figure 48.

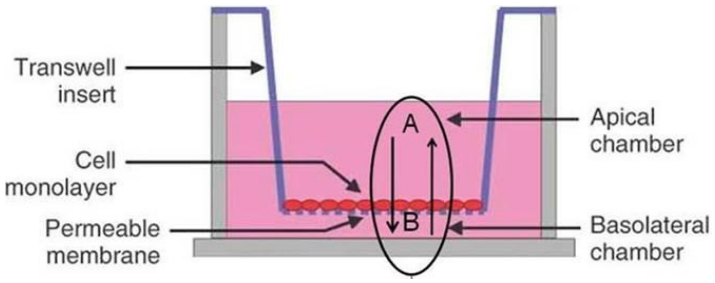


Figure 48: Schematic diagram of transepithelial transport across Calu-3 cells

The selected model antibiotics were dissolved in transport buffer (HBSS). The concentration of drug solutions were chosen based on the toxicity of each antibiotic, the solubility of the antibiotic in HBSS and the limit of detection by

high performance liquid chromatography (HPLC). The volume of antibiotic solution in the apical compartment was 150 µl for A-B transport, while the receiver compartment was loaded with 350 µl of HBSS. For the B-A transport, 350 µl of antibiotic solution was added to the basolateral compartment and the receiver compartment contained 150 µl of HBSS.

Aliquot of samples were drawn from the receiver compartments at pre-determined time points (30, 60, 120, 180 min) and the sample volume was replaced with fresh pre-warmed HBSS.

The cell layer was then harvested for analysis of the drug content inside the cells according to the method previously described (Haghi *et al.* 2010). Briefly, the cells were harvested and lysed using CellLytic M Cell Lytis buffer, following a centrifugation at 10000 *g* for 10 min. the supernatant was collected for the analysis for the cellular drug content.

4C 5 Sample quantification

Sample concentrations were determined by HPLC. A Shimadzu Prominence UFLC system equipped with an SPD-20A UV-Vis detector, LC-20AT solvent delivery unit, SIL-20A HT Autosampler (Shimadzu Corporation, Japan) was used for the analysis. The column, wavelength and mobile phase composition for each antibiotic studied are shown in Table 10. Flow rate was 1.0 ml/min and the injection volume was 70 µl for all samples tested. The apparent permeability coefficients (P_{app}, cm/s) was calculated using equation (2):

$$Papp = \frac{dQ}{dt} \times \frac{1}{A} \times \frac{1}{C0} \qquad (2)$$

Where *dQ/dt* is the linear transport rate of the drug, *A* is the surface area of the cell monolayer and C_0 is the initial concentration in the donor compartment.

Table 10. HPLC analysis conditions of all antibiotics.

Antibiotics	**Composition of mobile phase (v:v, %)** **Column**	**Wavelength (nm)**
Ciprofloxacin HCl	Methanol: 0.1 M phosphate buffer (30:70 v/v) pH=3.35 NovaPak C_{18} (5 µm, 150 x 3.9 mm)	275
Moxifloxacin HCl	Acetonitrile: 0.1 M phosphate buffer (25:75 v/v) pH=4.6 XBridge C_{18} (5 µm, 150 x 4.6 mm)	307
Rifampicin	Methanol: Water (85:15 v/v) XBridge C_{18} (5 µm, 150 x 4.6 mm)	254
Doxycycline HCl	Acetonitrile: 0.1 M phosphate buffer (30:70 v/v) pH=3.35 XBridge C_{18} column (5 µm, 150 x 4.6 mm)	350
Azithromycin dihydrate	Methanol: 0.03 M phosphate buffer (80:20 v/v) pH=9.5 XBridge C_{18} column (5 µm, 150 x 4.6 mm)	210
Tobramycin	Derivatization method according to *Pharmacopoeia* (USP 34)	365

4C 6 Statistical analysis

Measurements were performed in triplicate, unless differently stated. Values expressed as mean of at least three experiments with three replicates each ± SD. Statistical differences between the treatments and the controls were evaluated by the Student's t-test A (P values less than 0.05 were considered statistically significant)

LIST OF ABBREVIATIONS

P_{app}	Apparent permeability
AA	Amino acid
ACI	Andersen cascade impactor
API	Active Pharmaceutical Ingredients
ABC	ATP-binding cassette
AZT	Azithromycin dihydrate
BAL	Bronchoalveolar lavage
BCS	Biopharmaceutics Classification System
BrdU	Bromodeoxyuridine
BEBM	Bronchial epithelial basal medium
CPF	Ciprofloxacin HCl
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
d_{ae}	Aerodynamic diameter
DXC	Doxycycline HCl
DPI	Dry powder inhaler
DSC	Differential scanning calorimetry
DTPA	Diethylenetriaminepentaacetic acid
DMEM	Dulbecco's modified Eagle's medium
ED	Emitted dose
ENaC	Epithelial sodium channel
ERK	Extracellular signal-regulated kinase
FEV1	Forced expiratory volume in one second
FPD	Fine particle dose
FPF	Fine particle fraction
G	Gentamicin sulfate
HEC	Hydroxyethylcellulose

HBSS	Hanks balanced salt solution
HFA	Hydrofluoroalkanes
IL-8	Interleukin-8
IPA	Isopropyl alcohol
Klys	Ketoprofen lysine salt
Leu	L-leucine
LogP	Partition coefficient
LLS	Light laser scattering
LTB4	Leukotriene B4
MAPK	Mitogen-activated protein kinase
MMAD	Mass median aerodynamic diameter
MXF	Moxifloxacin HCl
MW	Molecular weight
NSAIDs	Non-steroidal anti-inflammatory drugs
NF-κB	Nuclear factor-κB
Pa	*Pseudomonas aeruginosa*
PCL	Periciliary liquid
pMDI	Pressurized metered dose inhalers
RFP	Rifampicin
SEM	Scanning electron microscopy
SCF	Super critical fluid drying
SD	Spray drying
TBM	Tobramycin

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Mariateresa