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PULMONARY DRUG DELIVERY: IN VITRO PREDICTION OF LUNG ABSORPTION

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SUMMARY

Within the past few decades, the lung has received increasing attention as a target for local and systemic drug delivery (1, 2). Drug administration via the respiratory tract appears promising because of the peculiar anatomical and physiological characteristics of the lung: large surface area, thin alveolar epithelium, high vascularization and relatively low level of metabolic enzymatic activity (3, 4).

The optimal absorption characteristics of a pulmonary drug depend on the site of action. For systemically acting drugs, absorption from the lung determines the therapeutic effect profile (onset, intensity and duration of action). On the contrary, for locally acting drugs, the absorption process may determine the removal and consequently the termination of drug action in the lung, as well as the onset of any systemically mediated adverse effects. On the other hand, a very high lung retention is not targeted due to accumulation/toxicity issue (5, 6). Therefore, when designing drugs for pulmonary delivery, it is important to consider both lung-tissue retention and permeability in order to find the right balance between these two parameters (6).

In vitro cell culture models of absorptive epithelia could be helpful and useful in early phase of drug discovery for the prediction of in vivodrug permeability and drug absorption.Advantages of cell culture models based on continuous cell lines are well demonstrated by the large use of the Caco-2 model of the gastrointestinal epithelium so that Caco-2 cells are now definitely recognized as the gold standard for permeability and transport studies of orally administered drug (7, 8).

However, at present there is no lung equivalent of the Caco-2 cell line to serve as well-established and reliable in vitro model of the respiratory epithelium (9, 10). For this reason, in respiratory delivery, there is a need to have a functionally relevant, continuous and robust in vitro epithelial cell modelto be used as a permeability screening tool that is predictive of in vivo lung absorption and to establish reliable In vitro-In vivo (IVIV) correlation to guide drug discovery programs (7).

Recently, the continuously growing bronchial epithelial cells, Calu-3, are quite often chosen as a model for the pulmonary epithelial barrier, so, in the PhD project, foremost cell culture model was set up and validated to demonstrate that in our hand, air- interfaced cell layers exhibit morphological and bioelectrical characteristics proper of the native epithelium. Once validated, Calu-3 cell line was used to assess the permeability (Papp) of a set of 8 locally acting drugs of varying molecular size, charge, lipophilicity and polar surface area. The permeability of these compounds was determined also in the well-recognized Caco-2 cells absorption model. Thus, the in vitro permeability values both from Caco-2 and Calu-3, were correlated with several in vivo pharmacokinetic (PK) parameters obtained after an intratracheal administration in rats to find out if

an IVIV correlation could be established. Overall, the data demonstrate that Calu-3 cells exhibit many of the features of primary cells, forming a uniform layer of polarized, well-differentiated cells with mucus secretion, functional tight-junction and important transporter proteins, confirming its potential use as a model to assess lung permeability.

Moreover, when Papp values determined both in Calu-3 and Caco-2 cell lines, were compared to in vivo absorption data in rats, a strong and reliable IVIV correlation was established with three in vivo PK parameters: lung drug half-life (T ½), lung mean residence time (MRT), and the percentage of drug retained in the lung at 24h after dose. Commonly, permeability data from cell culture, offer a convenient reproducible and quantifiable way to evaluate the absorption potential of a compound, leading to a rank order that highlights qualitatively those compounds that show favourable permeability characteristics. In the present study, it was demonstrated how Calu-3 and Caco-2 permeability data can be applied to quantitatively predict the extent of absorption in vivo. In future, in vitro Papp couldbe used to guide drug discovery programs allowing prediction and estimation of lung kinetic of inhaled drug.

To deep analyse the predictive power of Caco-2 and Calu-3 cell lines for permeability studies in pulmonary drug delivery, the two cell models were evaluated and compared to each other through a novel method of in vivo prediction named physiologically-based pharmacokinetic (PBPK) modelling. To this aim, routine ADME (Absorption, Distribution, Metabolism and Excretion) in vitro assays as well as non-standard in vitro assays were performed in order to provide all the in vitro experimental data and input information to fill in the PBPK model built in rat preclinical specie. As in vitro permeability data, both Caco-2 and Calu-3 Papp values were used to find out if a better simulation was achieved with one of the two cell lines. For all analysed compounds, the concentration-time curves in lungand plasma, generated starting from Calu-3 Papp values, were more close the actual PK profile. In the same way, also the estimation of key lung PK parameters was better achieved with Calu-3, since the ratio between the observed versus simulated parameter is often more proximal to unity in comparison to Caco-2 simulation.

Overall these results showed that Calu-3 cells line used in a PBPK modelling gave the right and proper input parameters to better describe the absorption process of pulmonary drug, leading for some compounds, to an improvement in prediction in comparison to Caco-2 and even better, for other compounds, making predictionpossible.

In future, application of PBPK modeling used in conjunction with IVIV correlation approach can provide a useful tool to study pulmonary drug disposition and enhance understanding of drug transport in the lung.

CHAPTER I

1. INTRODUCTION

1.1 PULMONARY DRUG DELIVERY

Until today, oral application of drug is the most preferred way to deliver a pharmaceutically active compound to its site of action. However, the first choice of delivery is not always possible, due to some restricting physicochemical or pharmacological properties of the drug (11).

In this situation, alternative routes have to be looked for, such as for examples, drug delivery via the skin or via the lung.

For several decades, the benefits of pulmonary drug delivery have been appreciated for treatment of respiratory disease like asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis and pulmonary infections.By direct targeting of drugs to the lungs, a high local concentration at the target site, rapid onset of action, lower systemic exposure and consequently reduced side effects can be achieved (12).

In recent years, the use of the inhaled route outside the respiratory therapeutic area, has found excellent acceptance and has attracted much interest as promising alternative route for systemic administration of drugs(11, 12).This can be easily understood considering the large surface area for drug absorption, the extensive lung vasculature, the thin air-blood barrierand the relatively low metabolic activity of the lung. Moreover, as it is a non-invasive “needle-free” delivery system, this route provides other advantages such as the avoidance of first pass metabolism in the liver and the overcome of poor gastrointestinal absorption (13).

However, the development of safe and effective medicines for administration via the lung requires a deep understanding of the kinetics and dynamics of drug in this biologically and physiologically complex system.

1.2 ANATOMY OF THE LUNG

The lungs is a complex organ system designed to enable the efficient exchange of gases between the blood and the respiratory airspace.

Functionally, the lung can be divided in two distinct zones: the conducting airways and the respiratory region (Fig.1).

The conducting airways (1-2 m^2, a relatively small surface area)consist of the air-transmitting passages of the nose, pharynx, larynx, trachea, bronchi and bronchioles. The main function of this region is to provide filtration, warming and humidification of inhaled air (14).

The epithelial lining of conducting airway epithelium is comprised of three major cell types: ciliated cells, secretory cells (mucous, goblet, serous and Clara) and basal cells.

Gradual changes in the structure of the conducting airways occur as the diameter of the respiratory tubes becomes smaller. The epithelium gradually changes from being pseudostratified and ciliated with goblet cells and submucosal glands interspersed to becoming a flattened squamous epithelium devoid of globetcells or glands (Fig. 2) (15).

Ciliated cells constitute the major cell population in the conducting airway epithelium, with their major function being the removal of inhaled particulate matters trapped in the mucus.

Secretory cells comprise 15-25 % of cells epithelium and are present in several forms. Mucous cells, also known as goblet cells, are the main producers of mucus. Mucus is a natural glycoprotein-hydrogel composed mainly of water (95%), glycoproteins (mucins) (2%), proteins (1%), inorganic salts (1%) and lipids (1%). Its thickness varies along the conducting airways being about 8 μm in the trachea to 3 μm in the bronchioles (Fig.3).

Basal cells, the other major cells types in the airway epithelium, reside near the basement membrane, interspersed between the taller ciliated and secretory cells, not directly in contact with the airway lumen. Basal cells are considered as the stem cells or progenitor cells for differentiated ciliated and secretory cells (Fig.2).

The respiratory region, where gas exchange takes place(80 - 120 m^2, a very large surface area), is composed of respiratory bronchioles, the alveolar ducts and the alveolar sacs.

The alveolar epithelium consists of the alveolar type I cells (AT-I), that cover 90% of the alveoli surface area, the small and cuboidal alveolar type II cells (AT-II) that cover 3% of the alveolar surface area, and the alveolar macrophages (12,13, 15). These three cells have different functions: AT-I cells provide short diffusion path for gas exchanges, ions and protein transport. However, these cells are unable to divide. Thus, AT-II cells have the ability to differentiate into AT-I cells; a

process that constitutes the replacement and repair mechanism of the epithelium in case of lung injury (15, 16). AT- II cells produce and secrete the lung surfactants (phospholipids and proteins) that decrease the surface tension in the alveoli and prevent alveolar collapse. Finally, the alveolar macrophages, as part of the immune system, reside on the surface of the alveolar epithelium, patrolling the lungs against foreign materials.

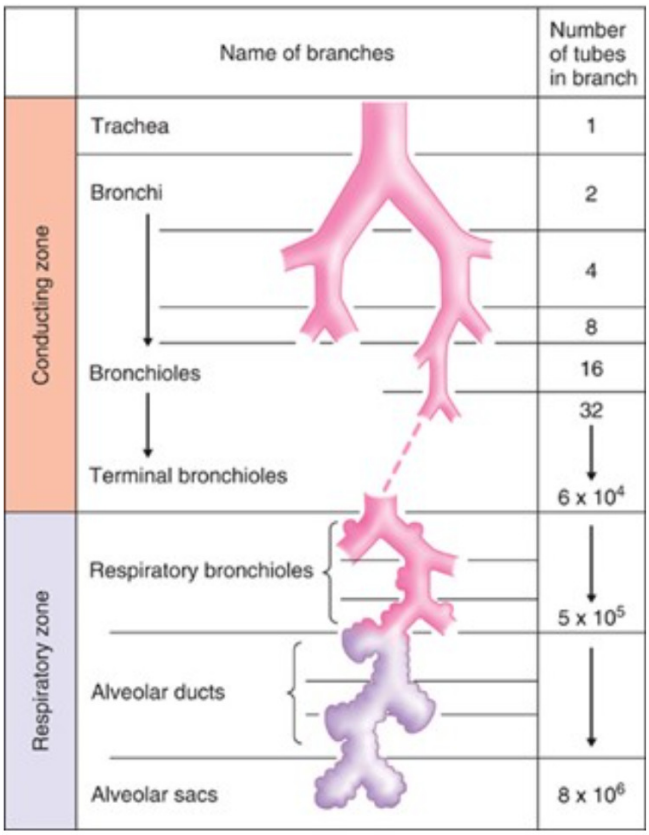


Fig. 1 Structure of the airways.

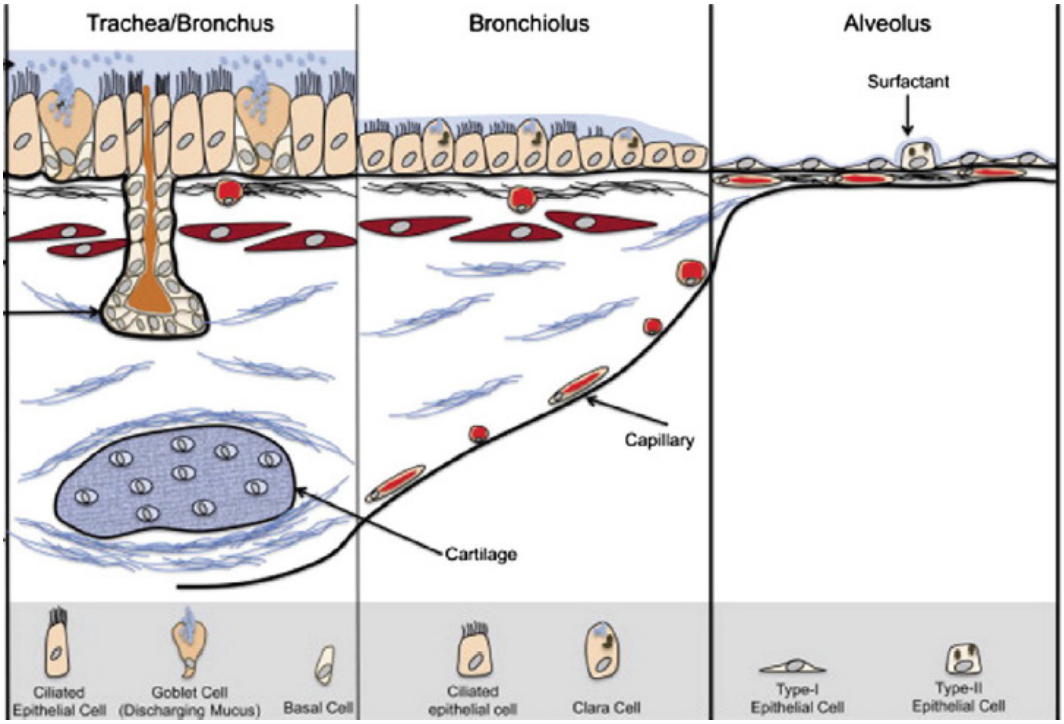


Fig.2. Different composition of lung epithelial cells in respiratory tract.

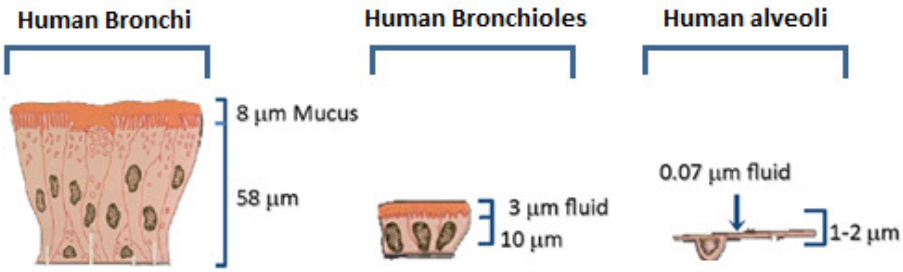


Fig.3.Lung regional differences in epithelial lining fluid thickness.

1.3 BARRIER FUNCTION OF THE RESPIRATORY TRACT

As one of the primary interfaces between the organism and the environment, the respiratory system is constantly exposed to airborne particles, potential pathogens and toxic gases in the inspired air.
As a result, a sophisticated respiratory host defence system, present from the nostrils to the alveoli, has evolved to clear offending agents (15). The system comprises mechanical (i.e. air filtration, cough, sneezing and mucociliary clearance), chemical (antioxidants, antiproteases and surfactant lipids) and immunological defence mechanisms (alveolar macrophages).
The first obstacle to particle penetration in the lung is the airway geometry, where the repeated bifurcations present a defensive "sifting" zone to trap particles via inertial impaction and sedimentation while allowing inhaled gases to be humidified and warmed to body temperature.
The barrier function of the airways is accomplishedalso by the lung epithelia that are barriers to drug transport to underlying tissues as well as to bloodstream (1). The defence is mediated via the integrity of the airway epithelium, maintained by several cell-cell adhesion mechanisms. The desmosomes and the intermediate junctions are involved in cell-cell adhesions. The tight junctions are narrow, belt like structures surrounding each cell at the apical pole (14, 15). The tight junctions are highly dynamic structures that act as barrier to fluid flow and control the transport of ions and solutes through the intercellular space.
The pulmonary barrier is also comprised of important extracellular element: the mucociliary escalator system. Inhaled particles are cleared from the airways through trapping of the particles in mucus upon deposition and subsequent clearance of the mucus, which is propelled by the coordinated beating of cilia towards the throat where it is swallowed or expectorated.
Moreover, to provide protection against potentially injurious agents, which enter the respiratory tract from the environment during inspiration, the bronchial epithelium secretes a number of mediators, including antibacterial, anti-proteases and anti-oxidant molecules.
Another defence mechanism in the lung are the alveolar macrophages found on the alveolar surface. These phagocytic cells play important role against bacteria and particles that have reached the alveoli. Phagocytosis results in clearance via a combination of slow enzymatic disposaltransport along the alveolar surface to the mucociliary escalator and translocation to the interstitium and tracheobronchial lymphatic capillaries.
From a drug delivery perspective, these components of the host defence system comprise barriers that must be overcome to ensure efficient drug deposition, lung permanence or absorption from the respiratory tract.

1.4 PULMONARY DRUG ABSORPTION

Once the drug is aerosolised in the respiratory tract, the immediate fate of the drug depends on several processes occurring onto the lung surface (Fig.4).

The first of these steps is the particles deposition. The deposition in the airways is controlled by particle properties (size, shape, density and charge), respiratory tract morphology and the breathing pattern (airflow rate and tidal volume) (11).

These parameters determine not only the quantity of compounds deposited, but also in what region of the respiratory tract the particles are deposited. The most important mechanisms of deposition are inertial impaction, gravitational sedimentation and Brownian diffusion (Fig.5).

Inertial impaction occurs predominantly in the extra-thoracic airways and in the tracheobronchial tree, where the airflow velocity is high and rapid change in flow direction occurs (1).

Generally, particles with a diameter larger than 10 μm are most likely deposited in the nasal and oral passages, pharynx and larynx; whereas 2 to 10 μm are most likely deposited in the tracheobronchial tree by inertial impaction. Gravitational sedimentation is of greatest importance in the small airways and alveoli and is most pronounced for particles with diameter of 0.5-2 μm. Ultrafine particles (≤ 0.5 μm) are deposited mainly by diffusional transport in the small airways and lung parenchyma where there is a maximal residence time of inspired air (Tab.1) (1).

Once deposited onto the lung surface, solid drug particles need to be wetted and dissolved before they can exert therapeutic activity.Essentially the dissolution rate is proportional to the drug's solubility and to the area of the solid-liquid interface. The solubility of a drug depends on the compound, the formulation and physical form of the drug as well as on the composition of the dissolving media in the lung: the epithelial lining fluid. The composition of this fluid in conducting airway is mainly water, salts, phospholipids and proteins, while the surface-lining layer in the alveoli is composed of a thin layer of surfactant (phospholipids and proteins). The thickness of the lining fluid varies from about 5-10 μm in the conducting airways and gradually decreases distally to about 0.01- 0.08 μm in the alveoli (Fig.3) (6). Thus, a drug particle deposited in the upper airways can be immersed in the lining fluid, while the lining fluid film may be much thinner than the diameter of a deposited drug particle in the alveoli. Consequently, the area of the solid-liquid interface between the particles and the fluid is proportional to particle surface area in the conducting airways but limited by the thickness of the fluid in the alveoli. This suggest that particles deposited in the upper airways could dissolve more rapidly than particles deposited in the alveoli. However,

other factors such as greater solubility, larger total interfacial surface area and more rapid absorption in the periphery could arguably lead to the opposite.

Following deposition and dissolution, aerosol drugs are absorbed through the pulmonary membrane barriers by passive diffusion and via transporters.

Lastly, drug absorbed from the air spaces into the blood, must traverse a final barrier: the endothelium, the cell monolayer that makes up the walls of the microvessels prior to reach systemic circulation.

Thus, in pulmonary delivery, the process of lung absorption depends on several factors including regional deposition, drug solubility and dissolution into the epithelial lining fluid and on permeability through lung epithelium relative to pulmonary clearance process (2,17) (Fig.6).

Also the binding to lining fluid (binding to protein and phospholipids) and the binding to lung tissue (binding to structures in the cell membrane, receptors, cell organelles and tissue proteins) could have different implication slowing down lung clearance and increasing local permanence (2,6,17).

Therefore, the optimal absorption characteristics of a pulmonary drug depend on the site of drug action. For locally acting drugs, the drug absorption process may determine the removal and consequently the termination of action of the drug in the lung, as well as the onset of any systemically mediated adverse effects. For systemically acting drugs, absorption from the lungs determines its therapeutic effect profile (onset, intensity and duration of action). For this reason, when designing drugs for pulmonary delivery, it is important to consider both lung-tissue retention and permeability, irrespective of site of action (7).

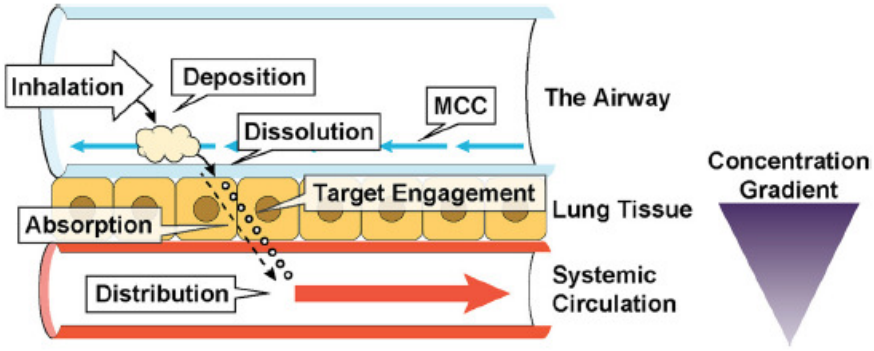


Fig.4.Lung disposition of pharmaceutical aerosol drugs following inhalation.

MCC: mucociliary clearance.

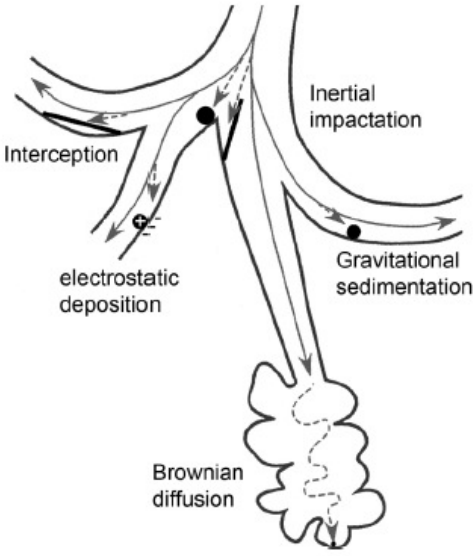


Fig.5Mechanism of particle deposition in the airways.

Location	Size	Mechanism
Primary Bronchi	5–10 μm	Impaction
Secondary Bronchi	1–5 μm	Sedimentation
Bronchioles	1–3 μm	Sedimentation
Alveoli	0.5–1 μm	Brownian motion

Tab.1 Correlation between the region of lung deposition, the particle size and the mechanism of deposition.

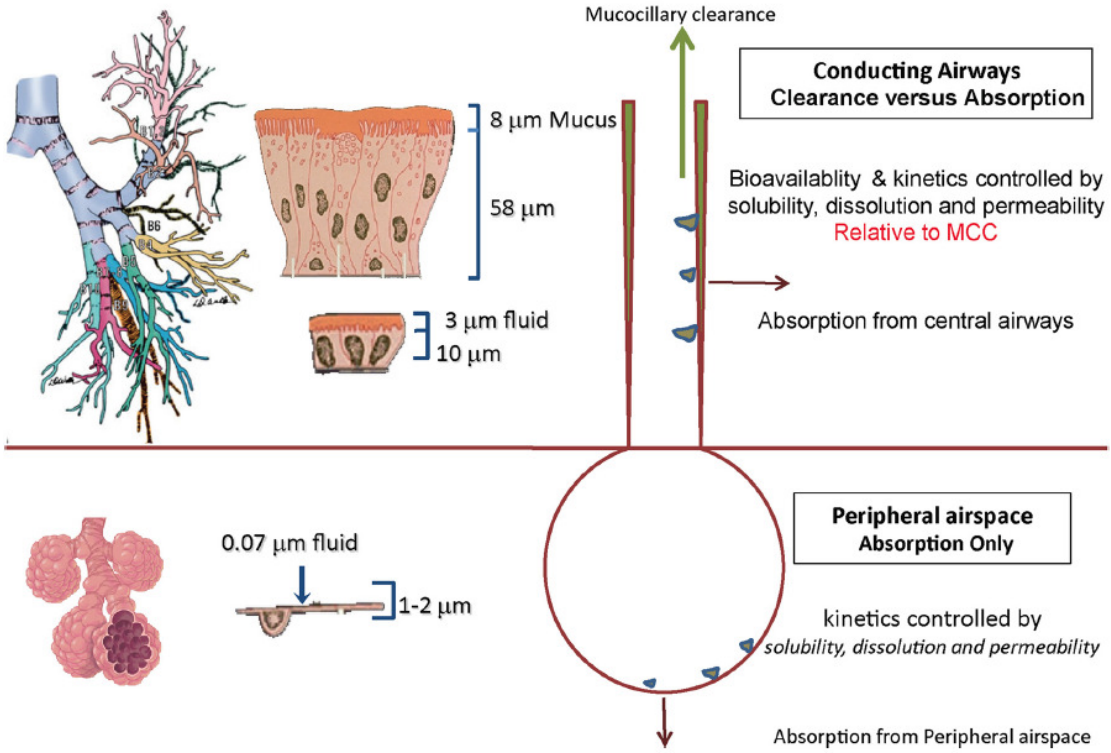


Fig.6 Schematic diagram of lung clearance and absorption process.

1.4.1 MECHANISM OF DRUG TRANSPORT ACROSS LUNG EPITHELIUM

The routes of drug absorption across the epithelium include passive and active transport mechanisms, involving paracellular and transcellulartransport, vesicular and active transport (Fig.7). The main process for drug absorption is by passive diffusion, a process driven by concentration gradient where a solute tends to pass from a point of higher concentration to a point of lower concentration.

The absorption of lipophilic compounds is generally considered to occur by membrane diffusion (transcellular) whereas hydrophilic solutes appear to be absorbed through the intercellular junction pores (paracellular) (6).

The large differencein the available area for free-diffusion (transcellular area>>paracellular area) may account for a rapid rate of transport for lipophilic drugs compared to hydrophilic drugs. (7)

Besides passive diffusion, transport across the cell membrane could be facilitated by transporters, which enhance uptake of compound into cells against a concentration gradient. Examples of drug-transporting proteins are members of solute carrier family (SLC): organic anion transporters (OAT and OATP) and organic cation transporters (OCT).

By contrast, drug transporters of the ATP-binding cassette family (ABC transporters) efflux compounds out of cells via an ATP-dependent mechanism. Examples of ABC transporters are breast cancer resistance protein (BCRP) and multidrug resistance proteins (MDR1 also known as P-glycoprotein P-gp).

P-gp is the predominant drug transporter, which acts to extrude a wide range of structurally and functionally unrelated drugs out of cells in which it is expressed. P-gp is found physiologically expressed at site such as the luminal membranes of endothelial cells of the blood-brain barrier, the blood testis barrier, epithelial apical membranes of intestines and proximal tubes of the kidney, the biliary canalicular membranes of hepatocytes and on the apical surface of pulmonary epithelial cells (10). This tissue and apical membrane specific localization coupled with its vectorial transport capacity serves to protect both vital organs and the organism as a whole,from potentially harmful xenobiotic exposure.

Membrane vesicles within the alveolar epithelial cell type I and II have been suggested to be involved in macromolecule transport across the alveolar epithelium. The main route of alveolar epithelial protein transport is though transcytosis involving caveolae and clathrin mediated endocytosis. This type of transport is of particular interest, especially for the increasing number of newly developed biopharmaceuticals, such us vaccines, monoclonal antibody-based products, and therapeutic peptides. From a pharmaceutical point of view, this class usually features huge sizes and poor permeability, in this way complicating their delivery.

Vesicular transport, efflux and uptake transport, work in concert with passive diffusion to influence the permeability of drugs through an epithelial cell layer, regulating drug distribution and being key determinants of drug absorption and clearance processes (6, 18).

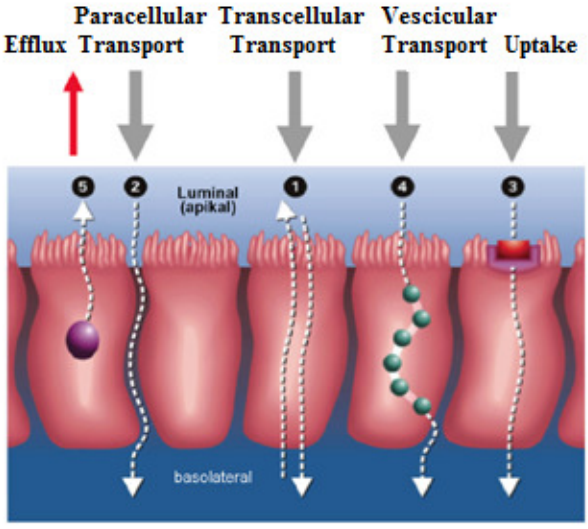


Fig.7Mechanism of drug transport across the lung epithelium.

1.5 IN VITRO MODELS FOR DRUG PERMEABILITY DETERMINATION

Measurement of drug transport across epithelial cell monolayers in tissue culture are routine assays performed in pharmaceutical research laboratories to predict the absorption properties of drug candidates, with application ranging from drug discovery and development process, to regulation of approved innovator and generic drug products (19).

Epithelial cell monolayers can be performed using primary or continuously growing cell lines (13). Primary cells are isolated directly from tissues of animals or humans, thus present cell characteristics and state of differentiation more similar to the in vivo situation, providing the closest in vitro representation of the native epithelium (1,13). However, the major limitations of primary cells are the large donor variability that gives an inherent variabilityto the system, the short life span of only few days in culture, the limited availability of tissue source and also the time consuming and costly isolation procedures (1, 13, 14,16).

Conversely, cell lines are usually obtained transforming primary cultures or are derived from tumours of various origin. Thesecells are able to grow indefinitely making reproducible culture possible with relatively easy standard procedures.

Therefore, the popularity of epithelial cell lines in studies of drug transport processes can probably be explained by the ease with which useful information are derived from these rather simple in vitro models providing a rapid, predictive and cost-effective approach to assess bioavailability across pharmacological barriers (10, 18).

For oral drugs, the human intestinal Caco-2 cell line, have been widely used as in vitro assay system to measure permeability and predict gastrointestinal absorption, so much that it is now considered the gold standard system for absorption studies (20).When cultured on porous membrane supports (transwell) (Fig.8), the ability of these cells to form a monolayer with tight junctions enables reproducible and biorelevant measurement of drug transport.

Caco-2 is an immortal human colon adenocarcinoma cell line that is able to differentiate in culture and resemble the epithelial lining of the human small intestine. Desirable aspects of this cell line are its morphology and the presence of multiple permeability mechanism. Caco-2, in fact, develops microvilli on its apical surface that resemble the morphology of gastrointestinal epithelial cells. Moreover, it also expresses cell membrane efflux transporters on the apical surface such us P-gp and BCRP as well as uptake transporters. This provides the opportunity to investigate various permeability mechanisms.

The information generated in this in vitro system, can generally be relatively easily translated into a good correlation and prediction of intestinal drug absorption, making possible the use of this cell culture as a screening tool in drug discovery programs for studies on structure-absorption relationship and for prediction of drug permeability (18).

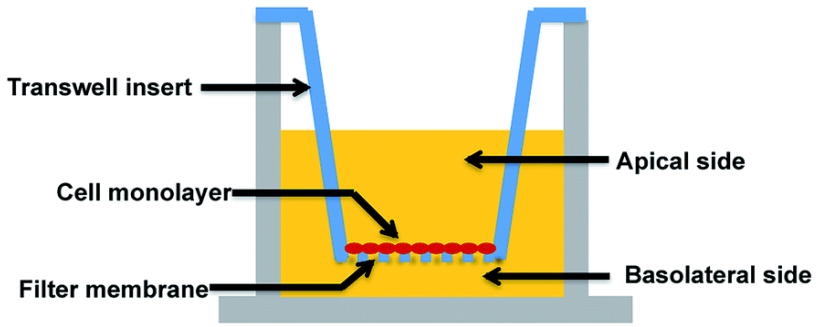


Fig. 8Transwell insert for cell culture and transport studies.

1.5.1 IN VITRO APPROCHES TO MODEL THE EPITHELIAL BARRIER OF THE RESPIRATORY TRACT

If for oral drugs, the human intestinal Caco-2 cell line have been widely used as in vitro assay system to measure permeability and predict gastrointestinal absorption, for pulmonary drug products, there is no lung equivalent of Caco-2 cells to serve as well-established and reliable in vitro model of the respiratory epithelium (9, 10).

In principle, the amper experience with intestinal cell culture Caco-2 appears easily transferable to field of pulmonary drug delivery, offering useful screening tool and alternative to animal testing. However, both the European Agency for the Evaluation of Medicinal Products (EMEA) and Food and Drug Administration (FDA) of United State of America recommend the use of monolayers of suitable and representative epithelial cells for classifying the permeability of drug compounds (21).

For this reason, in respiratory delivery, there is a need to have a functionally relevant, continuous and robust in vitro cell line of airway epithelium to be usedas a permeability screen tool that is predictive of in vivo lung absorption (7).

Already back in the 1980s, attempts have been made to simulate the respiratory epithelium using isolated organs or organ slices. However, these approaches have been limited by functional breakdown of the tissue, lacking reproducibility and high costs involved (22, 23, 24).

Finally, progress in cell culture overcame these issues and the continuously growing bronchial epithelial cells, Calu-3, are now quite often chosen as a model for the pulmonary epithelial barrier thanks to their easy availability, handling and to the ability of develop tight and polarized monolayers suitable for transport studies (7,10).

Calu-3 cells line is derived from human bronchial adenocarcinoma; when culture on permeable supports, it exhibits many of the features of primary cells displaying a polarized well-differentiated epithelium with essential drug transporter proteins and functional tight-junction.

In addition, when grown without a fluid filled in the apical compartment, the so called “air liquid interface” (AIC), Calu-3 cells form a pseudo-stratified layer of columnar cells, display enhanced ciliogenesis, secrete a tick mucous gel layer on the epithelium surface and possess a superior barrier integrity typical of the upper airway, leading to a good representative model of the human pulmonary barrier.(7, 10, 25, 26).

For the alveolar region, up to now, no immortalized cell line with sufficient barrier properties is available. Primary cells isolated from normal human lungs are able to reproduce the native alveolar

epithelium but are less reproducible and more time-consuming to work with compared to a cell line, which make them less suitable for permeability screening purposes(1, 10, 13, 16).

As outlined above, the respiratory epithelium is composed of a variety of different cell phenotypes, thus, choosing the right model to study drug transport in vitro is quite challenging (10).

However, besides the mentioneddifficulties to find arepresentative alveolar cell line, the increasing interest in bronchial Calu-3 cellsas reliable model for studying pulmonary drug delivery, is mainly due to the fact thatthe major site of lung deposition from current pharmaceutical aerosol devices, is the upper airways, where the epithelium is the principal barrier to drug absorption.

To effectively predict the fate of compounds delivered to the lung during the development and formulation process, a model of the airway epithelium reflecting the extent of drug permeability in vivois required. Thus, this model should accurately represents the barrier properties of the bronchial region in vivo for both passively absorbed and actively transported compounds, capable of measuring drug transport rates across the respiratory epithelium and predicting bioavailability at the level of the lung (10).

1.6 PREDICTION OF PHARAMCOKINETICOFPULMONARY DRUG

A key goal in pharmaceutical industry is a good understanding and prediction of the in vivo performance of a drug candidate. Prediction of human pharmacokinetics (PK) and disposition properties of new chemical entities from preclinical data has become a fundamental step in drug research and development process, because the exposure of drugs to target organs is the major factor determining their pharmacological and/or toxicological activity (27).

For this reason, in the recent years, several methods and approaches have been exceedingly increased in order to predict the PK and the dynamics of drug absorption, distribution, metabolism and elimination (ADME) through the body(11,28).

However, in pulmonary drug delivery, despite many technologies have been realizedto improve the formulation of inhalers, the fate of drug once deposited within the lungs, is still poorly understood (13, 29). The inhaled route of delivery is often limited by missing data regarding pulmonary disposition, nevertheless modern capabilities in imaging, analytical and biological science, make measurements of drug disposition and mode of action more accessible.

This is one reason that to date only few drugs and excipients are approved for pulmonary application by the regulatory authorities and this situation decelerates the development of modern inhalable medicines (30).

A better understanding of pulmonary PK and pharmacokinetic-pharmacodynamics (PK/PD) relationships would help mitigate the risk of not engaging successfully or persistently with the drug target as well as identifying the potential for drug accumulation in the lung or excessive systemic exposure.

Innovativein vitro models and predictive tools must therefore acknowledge the complexity of the interplay between the lung, the inhalation manoeuvre and pulmonary deposition that is difficult to simulate (30).

1.6.1 IN VITRO IN VIVO CORRELATION

One of the challenges of biopharmaceutics research is correlate in vitro drug information with in vivo drug profile through the In Vitro-In Vivo correlation (IVIVC) approach.

IVIVC is a predictive mathematical model describing the relationship between a specific in vitro property and a relevant in vivo response (7).

To establish a good correlation, in vitro and in vivo data are treated scientifically in order to explore the potential connection between the two parameters. Relationship, which could be linear or non-linear, implies a non-causality link between in vitro and in vivo data.Practically, once established, a good correlation is a tool for predicting in vivo results based only on in vitro information (31, 32, 36).

IVIVC could be applied in various areas and stages of drug research and development process.

Concerning drug absorption, the permeability coefficients estimated across monolayers of Caco-2 cell line, are commonly used to predict the absorption of orally administered drugs. In fact, if the permeability of Caco-2 is plotted as a function of the absorbed fraction after oral administration in humans, a good correlation between these two parameter is found (Fig.9). Once this curve has been established in a research laboratory, the in vitro permeability of a new compound in Caco-2 cells can be used to predict oral absorption in humans (8).

In this case, Caco-2 cells model and the respective IVIVC established,can play a key role in drug development and optimization process of oral dosage forms (31).

In pulmonary drug delivery, at present, there is no lung equivalent of Caco-2 cell line to serve as well-established and reliable in vitro model of the respiratory epithelium (9, 10).

For this reason, there is a need to have a functionally relevant, continuous and robust in vitro epithelial cell model to serve as permeability screen tool that is predictive of in vivo lung absorption and to establish reliable In vitro-In vivo (IVIV) correlation to find drug permeability parameters to guide drug discovery programs (7).

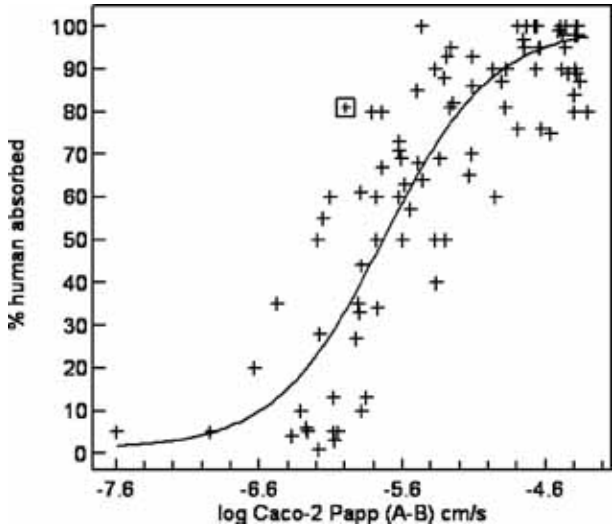


Fig. 9Comparison of the apparent permeability coefficients of different drugs measured in the Caco-2 cell monolayer and the fraction absorbed in humans.

1.6.2 PBPK

Integral to the In Vitro-In Vivo extrapolation approaches, a new method named physiologically-based pharmacokinetic (PBPK) modelling is rapidly gaining importance in pharmaceutical industry thanks to an increasing awareness of its powerful tool in predicting drug disposition in humans (33). PBPK modelling are simulation platforms used to model drug pharmacokinetic properties using routinely generated in vitro-in vivo data. It helps simulate the concentration-time profile of a drug in a specie by integrating the physicochemical properties of the compound with the physiology of the specie.

It consists of compartments corresponding to different tissues in the body connected by the circulating blood system (34). Each compartment is defined by a tissue volume or weight and tissue blood flow rate which is specific to the species of interest. Typically, these compartments include the main tissue of the body namely liver, kidney, lung, gut, spleen, skin, brain, adipose and bone.

Initially PBPK simulations are performed using compound-specific physicochemical properties and animal ADME in vitro data allowing a prediction of the in vivo PK profile of the drug in preclinical species. Data obtained from this simulation are then compared with the actual preclinical in vivo data, in order to validate the assumptions of the model. If the simulated profiles are able to recover the animal in vivo drug exposure, then different species can be modelled by simply replacing the physiological parameters with those for the species of interest or by allometric scaling, enabling the prediction of the drug disposition in human (Fig.10) (33,35).

Application of PBPK modelling used in conjunction with IVIVC can provide a useful and precious information during pharmaceutical research and development.

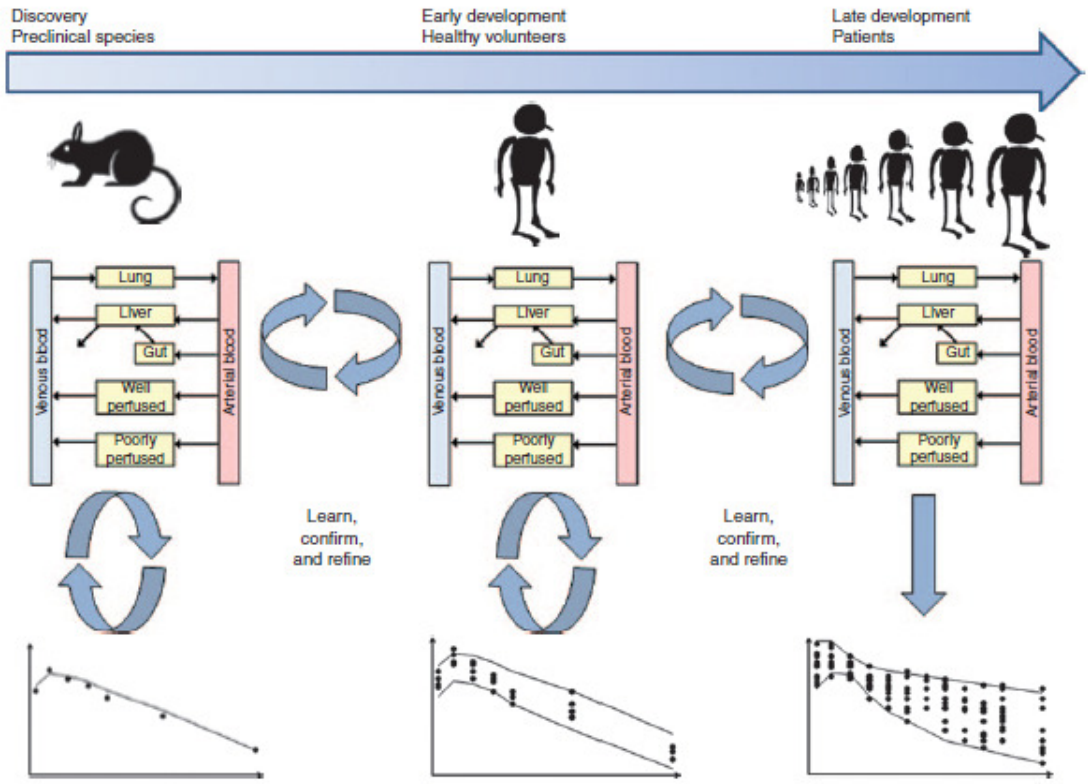


Fig.10PBK modeling strategy in drug discovery and development

1.7 AIM OF THE RESEACH

In vitro cell culture models of absorptive epithelia could be helpful and useful in early phase of drug discovery for the prediction of in vivodrug permeability and drug absorption.Advantages of cell culture models based on continuous cell lines are well demonstrated by the large use of the Caco-2 model of the gastrointestinal epithelium so that Caco-2 cells are now definitely recognized as the gold standard for permeability and transport studies of orally administered drug (7, 8).

However, at present, there is no lung equivalent of the Caco-2 cell line to serve as well-established and reliable in vitro model of the respiratory epithelium (9, 10).

Therefore, the goal of this study was to evaluate the potential of bronchial human Calu-3 cells asa permeabilityscreening tool predictive of in vivo lung absorption through the establishment of areliable In vitro-In vivo correlation and a more precise and accurate PBPK modelling simulation (7).

For this purpose, foremostthe cell culture model was validated to demonstrate that in our hand, air-interfaced cell layers exhibit morphological and bioelectrical characteristics proper of the native epithelium.

Once validated, Calu-3 cell line was used to assessthe permeability (Papp) of a set of 8locally acting drugs of varying molecular size, charge, lipophilicity and polar surface area.

The permeability of these compounds was determined also in the well-recognized Caco-2 cells absorption model.

Thus, the in vitro permeability values both from Caco-2 and Calu-3, were correlated with several in vivo PK parameters obtained after an intratracheal administration in rats to find out if an IVIV correlation could be established.

At last, to achieve a valuable prediction of PK profile through PBPK modelling, routine ADME in vitro assays as well as non-standard in vitro assays were performedin order to provide all the in vitro experimental data and input information to fill in the PBPK model built in rat preclinical specie.

As in vitro permeability data, both Caco-2 and Calu-3 Papp values were used to find out if a better simulation was achieved with one of the two cell lines.

CHAPTER II

2.MATERIALS AND METHODS

2.1 CACO-2 CELL CULTURE

Caco-2 cell line was purchased from the American Type Culture Collection (ATCC).
Cells between passages 55-65 were used in this study. Cell were grown in 75 cm^2 flasks in complete Dulbecco's Modified Eagle's Medium containing fetalbovin serum (10% v/v) , L-glutamine solution 200 mM (1% v/v) and Streptomycin (10/mg/ml) - Penicillin (10000 U/mL) (1% v/v) and maintained in a humidified atmosphere of 95% air and 5%CO_2 at 37°C. Cells were propagated according to ATCC recommendations.
When cultures reached approximately 80-90 % confluence, the cells were detached with trypsin-EDTA solution (0,5 g trypsin – 0,2 g EDTA/L) and were seeded onto Traswell insert (0.33 cm^2growth area)at a density of 5 x 10^5 cell/cm^2 in 200 µl apical and 700 µl basolateral medium. Media of both sides were removed 24h after seeding and cells were fed every alternative day with fresh apical and basolateral medium until day 18-23 of culture.

2.2 CALU-3 CELL CULTURE

Calu-3 cell line was purchased from the American Type Culture Collection (ATCC).
Cells between passages 45-50were used in this study. Cell were grown in 75 cm^2 flasks in Minimum Essential Medium Eagle containing fetalbovin serum (10% v/v) , L-glutamine solution 200 mM (1% v/v) and Streptomycin (10/mg/ml) - Penicillin (10000 U/mL) (1% v/v) and maintained in a humidified atmosphere of 95% air and 5%CO_2 at 37°C. Cells were propagated according to ATCC recommendations.

When cultures reached approximately 80-90 % confluence, the cells were detached with trypsin-EDTA solution (0,5 g trypsin – 0,2 g EDTA/L) and were seeded onto Traswell insert (0.33 cm^2 growth area) at a density of 4 x 10^5 cell/cm^2 in 200 µl apical and 700 µl basolateral medium.
To establish the air interface condition (AIC), the apical medium was removed 24h after seeding and cells were fed every alternative day with fresh basolateral medium only, until day 18-23 of culture.

2.3 IMMUNOSTAINING AND QUANTITATIVE POLYMERASE CHAIN REACTION FOR ASSESMENT OF LUNG EPITHELIAL MARKERS

To demonstrate that in our hands, Calu-3 layer exhibits the barrier properties and cells differentiation features of the bronchial epithelium, the expression of several differentiation and cell-adhesion markers was evaluated through immunostaining and quantitative polymerase chain reaction(qPCR).
In particular the presence of MUC5AC (expressed by globlet cells as a component of mucus), β-Tubulin IV (often expressed as cytoskeletal protein but apical expression is a commonly used marker of ciliated epithelial cells), ZO-1 (tight junction protein), E-Cadherin and Desmopakine (cell adhesion molecules and epithelial cell markers) was monitored at day 2, 9, 16 and 22 of AIC culture on Transwell.
For immunostaing, Calu-3 cells were fixed in situ on insert and transferred to glass slides for visualisation. For this purpose, medium was aspirated from the Transwell and cells were washed twice with Phosphate Buffer Saline (PBS). Cells were then fixed, blocked and permeabilised using Image iT FX kits (Invitrogen) according to instruction manual. Thus, cells were incubated with appropriate mouse anti human primary antibodies (ZO-1 dil. 1:50, β tubulin dil. 1:50,ZO-1 dil. 1:200,E-cadherin dil. 1:400, MUC5AC dil 1:100, all from Invitrogen) for 60 minutes at 37°C followed by goat AlexaFluor488 labelled anti mouse IgG secondary antibody (1:400) for 75 minutes at 37°before mounting. Cells nuclei were counter-stained with DAPI.
Controls were incubated with secondary antibody alone or primary isotype control antibody (IgG1 dil. 1:200) followed by secondary antibody.
Cells were visualized using Fluorescence Microscope (Nikon TI-E) and representative images were taken.

For qPCRanalysis, on the day on interest, cells cultured on Transwellwere lysed and mRNA was extracted using illustraRNAspin Mini RNA isolation kit (GE Healthcare) according to the manufacturer's protocol. cDNA was synthesized using Superscript II (Invitrogen) and random hexamer primers as per instructions. cDNA level were quantified using a series of TaqManassays (MUC5AC, β-Tubulin IV, ZO-1, E-Cadherin and DesmopakineTaqMan Gene Expression Assays from Lifetechnologies). Probes were labelled with FAM. qPCR was performed using TaqMan gene expression master mix (applied Biosystems) and carried out on a 7500 Real Time PCR Sequence Detection System (Applied Biosystems).

Data are normalized to the housekeeping gene β Actin and quantified using the 2-ΔΔCt method.

Changes of gene expression were elaborated by 7500 System Software (Sequence Detector Software Version 1.4) in an Excel file with the Comparative Ct Method, quantification approach also known as "2ΔΔCt Method" where relative expression level are calculated compared to a non-treated control sample used as a calibrator in each experiment (day 2 cell culture). The amount of target, relative to the calibrator and normalized to the reference gene, was measured by the Fold Regulation or relative quantities (RQ) that express the up or down regulation of the target gene in the sample of interest versus the control sample and was calculated by the following equation:

$$RQ = 2^{-\Delta\Delta Ct}$$

where:

Ct (Threshold cycle) = PCR cycle number at which the reporter fluorescence is greater than the threshold. The threshold value is determined automatically by the software program of the Real time PCR instrument and it is inversely proportional to the original relative expression level of the gene of interest

ΔΔCt = ΔCt sample – ΔCt calibrator where ΔCt is the Ct of the target gene subtracted by the Ct of the reference gene.

ΔΔCt thus represents the normalized expression of the target gene in the treated sample, relative to the normalized expression of the calibrator sample.

2.4 TRANSEPITHELIAL ELECTRICAL RESISTENCE OF CELL LAYER

Formation of functional tight junctional complex and attainment of confluent monolayers were monitored by measuring the transepithelial electrical resistence (TEER) that is a measure of the integrity of the cell layer.

For Calu-3 cell monolayers, TEER was monitored over time from day 2 to day 35 to find the proper culture period for transport studies.

Moreover, in order to assess the integrity of cell layers during the flux experiment, TEER was measured before and after each experiment with Caco-2 and Calu-3 cell line.

TEER was measured using an EVOM Voltohmmeter with STX-2 chopstick electrodes (Fig.11). Prewarmed fresh medium was added to the apical and basolateral sides of cell monolayer. Cells were then equilibrated for 30 minutes in a humidified atmosphere of 95% air and 5%CO_2at 37°C prior to resistance measurements. TEER was calculated by subtracting the resistance of a blank insert and corrected for the surface area of the Transwell according to the following formula:

$$\text{Resistance of a unit area} = \text{Resistance } (\Omega) \times \text{Membrane Area } (cm^2)$$

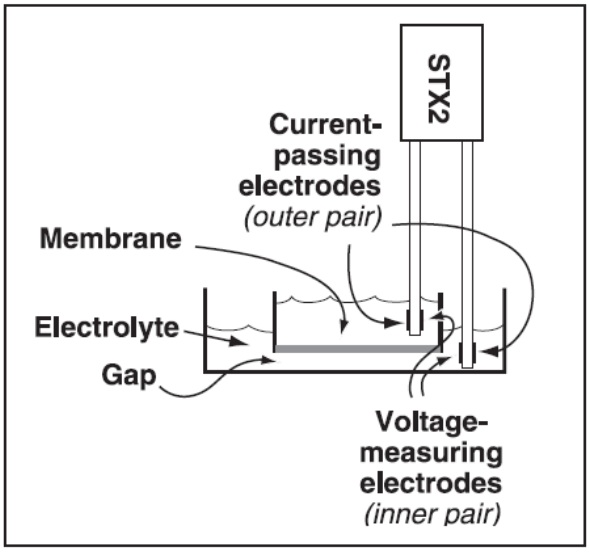


Fig.11 TEER measurement in Traswell insert

2.5 IN VITRO TRASPORT EXPERIMENTS

To validate the Calu-3 cell line as pulmonary permeability cell model, different reference compoundswere used: Propranolol and Metoprolol, known to have high epithelial permeability, Atenolol and Sulpiride, known to be low permeability compounds as well as Fluorescein, asparacellular drug transport marker. For these compounds, transport studies in apical to basolateral direction were conducted and the apparent permeability coefficient (Papp) was determined.

Once validated, the permeabilityof a set of 8locally acting drugs of varying molecular size, charge, lipophilicity, and polar surface area were estimated in Calu-3 cell line as well as in the well-established Caco-2 cells model.

Transport experiments were conducted using cells cultured on Transwell for 3 weeks, whenthe cells showed the optimal barrier properties, except for Fluorescein transport that was studied over a period from 2 to 35 days in culture to monitor the formation of tight junctional complex.

In order to assess the integrity of cell layers during the flux experiment, TEER was measured before and after each experiment(see Paragraph 2.4).

The transport was measured in apical to basolateral directionin triplicate. The donor solutions were made up in Hank's Balanced Salt Solution (HBSS) at concentration of 10 μM. Test solution were added to the donor chamber (apical) and fresh HBSS to the receptor chamber (basolateral).

Donor chamber were immediately sampled for determination of the initial compound concentration (C0). Cell layers were then incubated at 37°C in a humidified atmosphere of 95% air and 5%$CO_2$for 2 hours. At the end of incubation, samples were withdrawn from the receptor compartments for permeability determination as well as from donor compartment for recovery calculation.

As controls of the experiment, the permeability of reference compounds Sulpiride (low permeability), Propranolol (high permeability) and Digoxin (P-gp substrate) was assessed in each experimental session simultaneously.

The coefficient of apparent permeability Papp was calculated as follow:

$$Papp = (Vr/C0)\ (1/S)\ (dC/dT)$$

Where Vr is the volume of medium in the receptor chamber , C0 is the concentration of the test drug in the donor chamber at the beginning of the experiment, S is the surface area of monolayer, dC/dt is the linear slope of the drug concentration in the receptor chamber with time.

Recovery was calculated according to the following formula:

$$\text{Recovery \%} = \frac{(\text{C T120 d})(\text{Vd}) + (\text{C T120 r})(\text{Vr})}{(\text{C0})} * 100$$

Where (C T120 d) is the concentration of the test drug in donor chamber at the end of 120minutes incubation, Vd is the volume of medium in donor chamber. (C T120 r) is the concentration of the test drug in receptor chamber at the end of 120minutes incubation , Vr is the volume of medium in the receptor chamber , C0 is the concentration of the test drug in the donor chamberat the beginning of the experiment.
Recovery between 70 and 120% were considered accetable.

2.5.1 DIGOXIN BIDIRECTIONAL TRANSPORT WITH AND WITHOUT PGP INHIBITORS

Digoxin, a known substrate of Pgp (the major transporter involved in xenobiotic cell efflux), was used to verify the presence and the function of this protein on the apical membrane of Calu-3.
For this compound, transport studies were performed in both directions (Fig. 12), apical to basolateral (A/B) and basolateral to apical (B/A) in presence or in absence of a known Pgp inhibitor. Two different Pgp inhibitors were used: Quinidine and Verapamil.
Digoxin in transport buffer (10 µM) were added to the donor compartments of either apical (for A/B study) or basolateral chamber (for B/A study) of the insert system followed by incubation at 37°C in a humidified atmosphere of 95% air and 5%$CO_2$for 2 hours.
Donor chamber were immediately sampled for determination of the initial compound concentration (C0). Samples from receptor compartments were collected at the end incubation time as well as from donor compartment for recovery calculation.
In the inhibition study, cells were simultaneously incubated with Digoxin and a known P-gP Inhibitor at 10 µM (Verapamil or Quinidine).
Recovery, Papp A/B and Papp B/A were calculated as described above (see Paragraph 2.5).
Moreover, the Efflux Ratio (ER), that is the ratio between Papp B/A and Papp A/B were estimated with and without inhibitors. If the compound passes the epithelial barrier through simply passive diffusion, this ratio is around the unity. Instead, an ER value higher than 2 indicates a possible

efflux transporter mechanism. In the presence of a Pgp inhibitor, a significant reduction of ER (>50%) or to unity, suggests a possible involvement of the Pgp transporter.

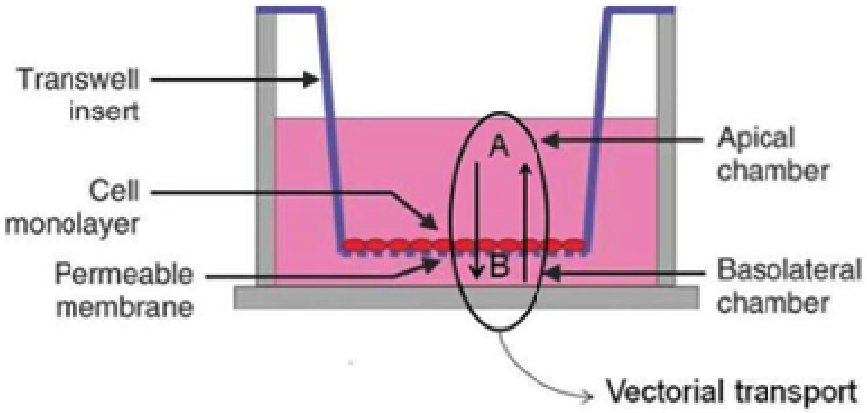


Fig. 12 Bidirectional Transport studies

2.5.2 SAMPLES QUANTIFICATION

Fluorescent samples of fluorescein transport experiments were measured in black, 96 well plates using a fluorescence plate reader (EnVision, PerkinElmer) at excitation and emission wavelengths of 485 and 520nm. Proper standard curves were prepared in HBSS for determination of fluorescein concentration both in donor and receptor compartments.

Samples of all other compounds were subjected to analysis for concentration determination by high performance liquid chromatography coupled with tandem mass spectrometry (HPLC/MS/MS).

In this work, samples were analysed by Waters AQUITY-XEVO TQD System and processed with Software MassLynx V4.1 (Waters). As stationary phase for Propranolo, Metoprolol and Atenolol, Luna 3µ CN 100A column (50 x2.00mm) was used. For all other compounds, Synergy 4u Fusion C18 - RP 80A, 75 x 2.00 mm was chosen. Mobil phase for Digoxin analysis was composed of 10 mM Ammonium Acetate in H_2O (A) and 10 mM Ammonium Acetate in methanol (B). Mobil phase for all other compounds analysis was H_2O + 0.1% Formic Acid (A) and Acetonitril + 0.1% Formic Acid (B). The following gradient program were used:

Gradient for Propranolo, Metoprolol, Atenolol analysis:

% A	% B	Flow (ml/min)	Time (min)
70	30	0.4	0
70	30	0.4	2
30	70	0.4	2.5
30	70	0.4	3.5
70	30	0.4	4
70	30	0.4	6

Gradient for analysis of all the other compounds:

% A	% B	Flow (ml/min)	Time (min)
95	5	0.4	0
95	5	0.4	1
40	60	0.4	2
40	60	0.4	3
20	80	0.4	4
20	80	0.4	5
95	5	0.4	6
95	5	0.4	11

For each compound, proper standard curves were prepared in HBSS for determination of compound concentration.

Same volumes from unknown samples and standard curves were collected and mixed to a fixed concentration of proper internal standard (IS).

The area ratio between analyte peak and internal standard were reported for data analysis.

2.6 SIMULATED LUNG FLUID (SLF) SOLUBILITY ASSAY, LUNG TISSUE AND SLF BINDING ASSAY

To achieve a valuable prediction of PK profile through PBPK modelling, routine ADME in vitro assays as well as non-standard in vitro assays were performedin order to provide all the in vitro experimental data and input information to fill in the PBPK model.

A series of non-standard in vitro assays including lung tissue binding (LTB), simulated lung fluid (SLF) binding and SLF solubility assay, were carried out in order to supply more in vitro data, useful in understanding the different processes and aspects of pulmonary drug exposure.

The composition of lining fluid in conducting airway is mainly water (96%), salts, phospholipids, proteins and mucins with a 6.9 pH.For this reason to simulate the lining fluid of the lung, a solution of PBS with 0.02% of Curosurf 80 mg/ml, a natural pulmonary surfactant preparation of porcine origin, was chosen.

Instead, for lung tissue binding, fresh tissue homogenates were prepared directly from rat lungsusing Precellys Evolution homogenator coupled to Cryolys temperature controller (Bertin Technologies, France). For the estimation of lung tissue binding and SLF binding, the equilibrium dialysis system, commonly used as standard method for plasma protein binding, was chosen.

The equilibrium dialysis system consists of two side-by-side chamber separated by dialysis membrane with a specific molecular weight cut-off that avoid protein-bonded compound to cross the membrane. Proper matrix (lung tissue homogenate and SLF) spiked with the compound of interest is added to one chamber of dialysis system (donor chamber) and buffer to the other chamber (acceptor chamber). Over time, only free compound diffuses from the donor chamber to the acceptor chamber until its concentration across the membrane is at equilibrium (Fig.13).

The amount of compound was then quantified in donor and acceptor chamber with HPLC-MS/MS technology as described above (see paragraph 2.5.2) and the percentage of binding to biological matrix was determined according to the following formula:

SLF BINDING

$$\% \text{ Free in SLF} = (\text{Conc. acceptor buffer chamber} / \text{Conc. donor SLF Chamber}) * 100\%$$

$$\% \text{ Bound} = 100 - \% \text{ Free}$$

LUNG TISSUE BINDING

$$\text{Bound fraction} = \frac{\text{Conc. donor homogenate chamber} - \text{Conc. Acceptor buffer chamber}}{\text{Conc. Donor homogenate chamber}}$$

$$\text{Unbound fraction } (f\text{u homogenate}) = 1 - \text{Bound fraction}$$

$$\% \text{ free lung } (f\text{u lung}) = \frac{(1/D) * 100}{((1/ f\text{u homogenate}) - 1) + 1/D}$$

$$\% \text{ bound lung} = 100 - \% f\text{u lung}$$

Where D= Dilution factor, which is 5

To investigate the solubility of selected compounds in SLF, a modified equilibrium dialysis procedure was used. In this case, the compound of interest was added as powder (in excess amount) in the donor chamber containing SLF and not as solution as in the case of SLF binding assay. Over time, only dissolved compound diffuses from the donor chamber to the acceptor chamber until the concentration in the acceptor chamber reached a plateau that represents the maximum solubility of compound in SLF.

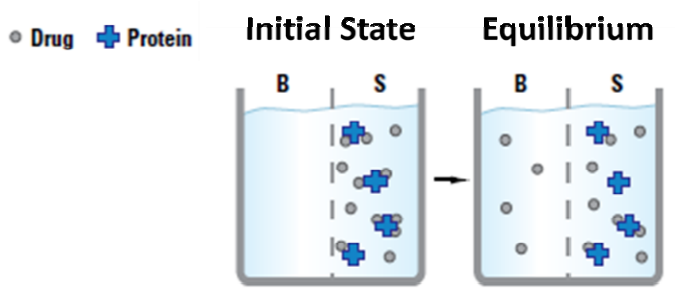


Fig.13 Schematic representation of Equilibrium Dialysis procedure

2.7 IN VIVO STUDY: RAT INTRATRACHEAL ADMINISTRATION

To achieve a more relevant IVIV correlation, the in vivo rat dosing procedure was performed through a novel technique: The DustGun. The DustGun technology allows generating a dry powder respirableaeresol that is inhaled during the spontaneous breath of the rat so as to mimic, as much as possible, the clinical inhalation exposure. Moreover, a novel dosing system,PreciseInhale dispensing system was used to improve the dosing precision through the measure of the aereosol concentration and the individual ventilation pattern of the exposed rat .

In this study, male CD rats weighing 300± 50 gr were used. For all the compounds, the target deposited lung dose of the aerosolized powders was 10 µg/lung. 1 mg of substance was loaded on the loading cylinder of Dustgun System, and then rat was anaesthetized by inhalation of Sevoflurane. Before intubation, rat was suspended in a supine position on a small table by the superior incisors. The tongue was moved to the side of the mouth using pincers in order to prevent obstruction of the trachea. The trachea was visualized using a laryngoscope and then a steel catheter was introduced until the trachea bifurcation. Then, rat was connected to the exposure block of the instrument and, before the exposure, it was monitored for few seconds to ensure a stable breathing.

The inhaled dose necessary for reaching the target dose in the lung was entered in the exposure control software. After aerosol generation, the valve was opened allowing the inhalation of the aerosol; then, the exposure was automatically terminated by the software when the entered inhaled dose is reached. Thus, the animal was removed from the intubation system, returned to its cage, and allowed to recover from the anesthesia.

Subsequently, animals were anesthetized and 1 ml of blood was collected through aortic punctureat 0.083-0.25-0.5-1-2-4-7-24-48 and 96 hours post-dosing. Blood samples were immediately transferred into heparinized polystyrene tubes. Plasma was separated by centrifugation at 1500 g for 10 min at 4 °C and stored at −80 °C until the analysis. After sample collection, animals were sacrificed by total bleeding through aortic cutting off and lungs and trachea were excised, washed with cold saline, weighed and stored at −80 °C for the analysis. Prior of the analysis, tissue samples were added with a mixture of saline solution/ACN 50/50 with a ratio of 3mL/g tissue. Then, lungs were homogenizedwith Precellys Evolution homogenator coupled to Cryolys temperature controller (Bertin Technologies, France).

The amount of compound in plasma and lung was then quantified with HPLC-MS/MS technology as described above (see paragraph 2.5.2)

2.8 IN VITRO IN VIVO CORRELATION

For the IVIV correlation, the apparent permeability coefficients estimated both in Caco-2 and Calu-3 cells line were correlated with different pharmacokinetic parameters that describe the drug disposition in lung:

- Lung drug half-life (T ½). It is the time taken for half of the dose to be eliminated from the lung.
- Lung mean residence time (MRT). It is the average time that the drug stays in the lung prior to elimination.

Both parameters characterize the elimination rate of compound however MRT is a time averaged parameter that applies throughout the entire concentration versus time profile. Half-life, on the other hand, typically describes the rate of decline for a particular phase of the concentration versus time profile (the apparent terminal elimination phase). If a compound shows multiphase kinetic, it has a different T ½ in each phase. In these cases, the terminal T ½ could represent only a limited percentage of drug elimination and can be much longer than the MRT. Therefore, for a multiphase compound, the MRT could better represent the effective elimination of the drug.

- Mean absorption time (MAT). This parameter denotes the average time it takes for a molecule to get absorbed into the system circulation. The longer the MAT, the greater the pulmonary residence. It is determined as difference between MRT in plasma after intratracheal administration and intravenous administration. It is an indicator of the lung absorption process since the plasma MRT difference between these two routes of administration is only due to absorption mechanism.
- Percentage of drug retained in the lung at 24h after dose.

Papp valuewas correlated with each of these in vivo PK parameters for the establishment of a reliable In vitro-In vivo correlation.

2.9 PBPK SIMULATION

The structure of the general PBPK model was based on the one reported by Poulin and Theil (Journal of Pharmaceutical Sciences, 2002). It consists of compartments corresponding to different tissues in the body connected by the circulating blood system. Each compartment is defined by a tissue volume or weight and tissue blood flow rate which is specific to the specie of interest. Only liver and kidney were considered as eliminating tissues for each compound.

For simulation, Matlab software was used; this platform comprises a series of differential equations that describe, as a whole, the dynamics of drug absorption, distribution, metabolism and elimination through the body.

All generated ADME in vitro data (Fig.14) were included in PBPK model built on rat physiological parameters tosimulate the drug concentration-time profile in plasma and lung after IT administration in rat. The simulations were then comparedwith the actual in vivo data in rats.

As in vitro permeability, both Caco-2 and Calu-3 Papp values were used to find out if a better simulation was achieved with one of the two cell lines.

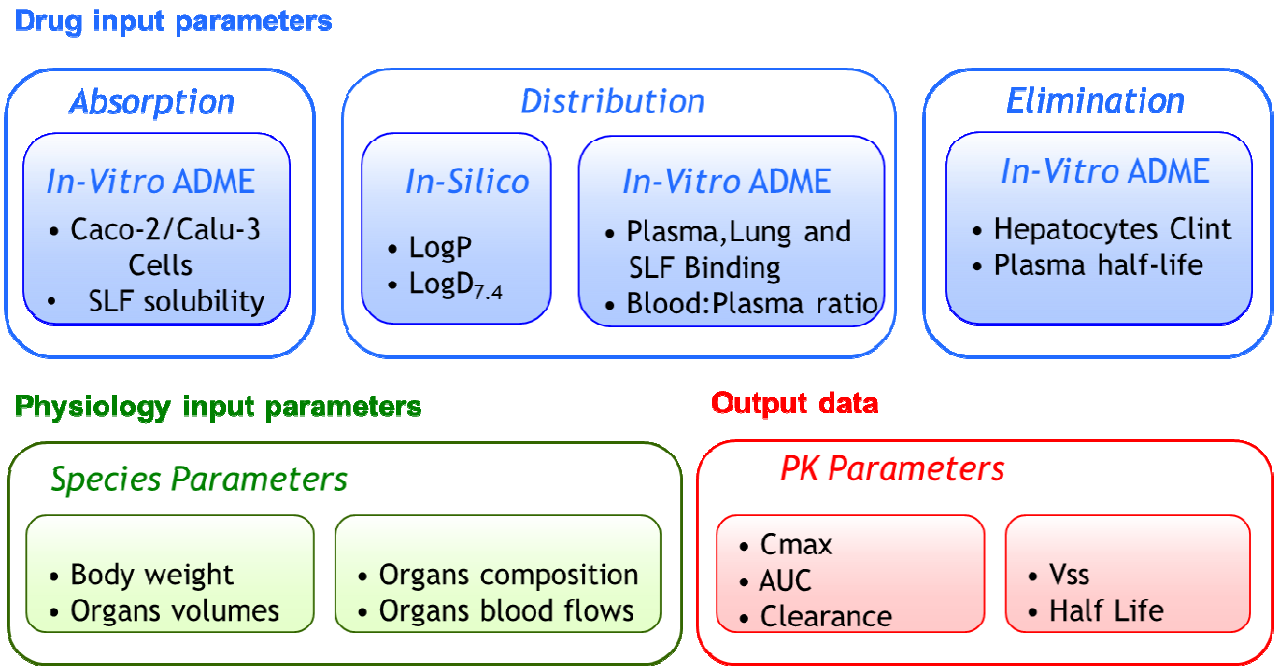


Fig.14 Input and output parameters for a PBPK modelling

CHAPTER III

3. RESULTS

3.1 CALU-3 CELL MODEL SET UP AND VALIDATION

To evaluate the potential of bronchial human Calu-3 cells as a permeability screening tool predictive of in vivo lung absorption, morphological and bioelectrical propertiesof cell line were investigated to verify thatcells exhibit features typical of the native epithelium.

3.1.1 INTEGRITY OF CELL LAYER

Calu-3 cell line were cultured, under air-interfeced condition on permeable supports,for up to 35 days and monitored for tight barrier properties proper of the upper airway epithelium.
First, the capacity to form tight junction was evaluated through the measure of the transepithelial electrical resistence (TEER) and then by the permeability estimation ofparacellular transport marker Fluorescein Sodium Salt.
As shown in Fig. 15, Calu-3 developed resistance as function of time reaching a TEER > 300 ohm-cm2 from day 16 up to day 35 of culture.
Moreover cells demonstrated a significant decrease in permeability of Fluorescein from day2 to day 35 reaching the expected Papp value < $2*10^{-6}$for low permeability compound on day 16 (Fig.16). This is consistent with the TEER measurement results described above.

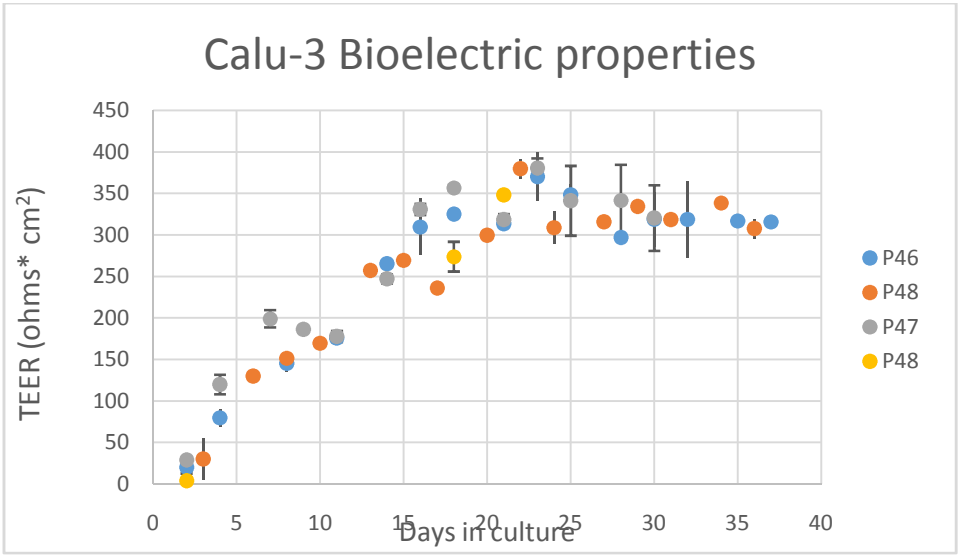


Fig. 15 Time course of transepithelial electrical resistance (TEER) development in Calu-3 cells. Data points are mean n=15. The different series are monolayers from distinct cell passages.

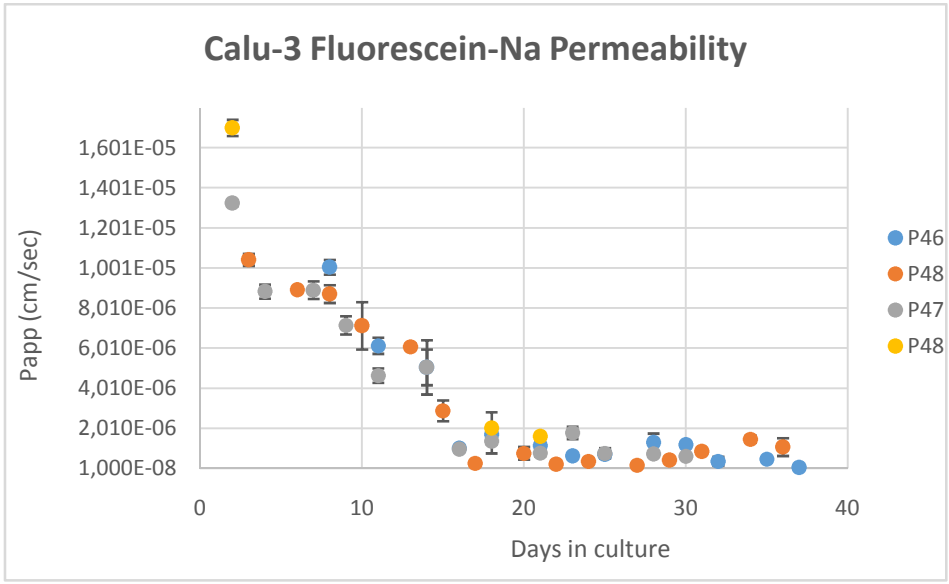


Fig.16Time course of permeability of paracellular marker (Fluorescein –Na) in Calu-3 cells. Data points are mean n=6. The different series are monolayers from distinct cell passages.

3.1.2 IMMUNOSTAINING AND QUANTITATIVE POLYMERASE CHAIN REACTION FOR ASSESMENT OF LUNG EPITHELIAL MARKERS

The expression of several differentiation and cell-adhesion markers was evaluated through immunostaining and quantitative polymerase chain reaction(qPCR). In particular the presence of MUC5AC (expressed by globlet cells as a component of mucus), β-Tubulin IV (often expressed as cytoskeletal protein but apical expression is a commonly used marker of ciliated epithelial cells), ZO-1 (tight junction protein), E-Cadherin and Desmopakine (cell adhesion molecules and epithelial cell markers) was monitored at day 2, 9, 16 and 22 of AIC culture on Transwell.

According to the results, a time dependent expression of selected markers was found.

Specifically, ZO-1, β tubuline and Desmopakine were found more expressed on day 16 of culture if compared with day 2, instead at day 22 the expression level was almost comparable with day 2 (Fig.17,18,19). MUC5AC and E-caderin expression in Calu-3 cells was time dependent, increasing with the days in culture (Fig.20,21).

Alsoimmunostaing analysis, performed at day 16, revealed an high expression of these protein in the cellular model (Fig.22).

Overall, these data suggest that the window for optimal barrier properties and so for permeability studies is between the day 16th and the 22th of cell culture.

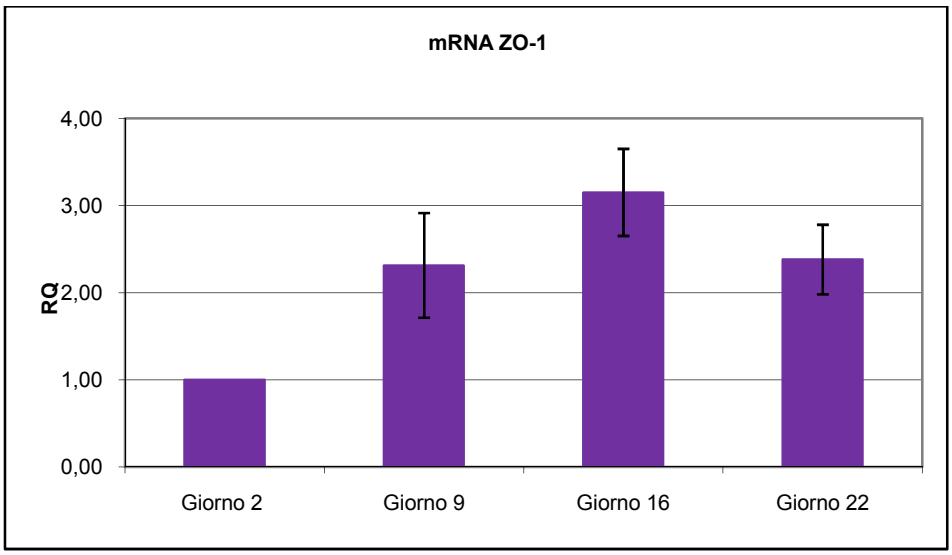


Fig.17 Zo-1 mRna expression level at different day of Calu-3 culture. Data are normalized to the housekeeping gene β Actin and quantified using the 2-ΔΔCt method.

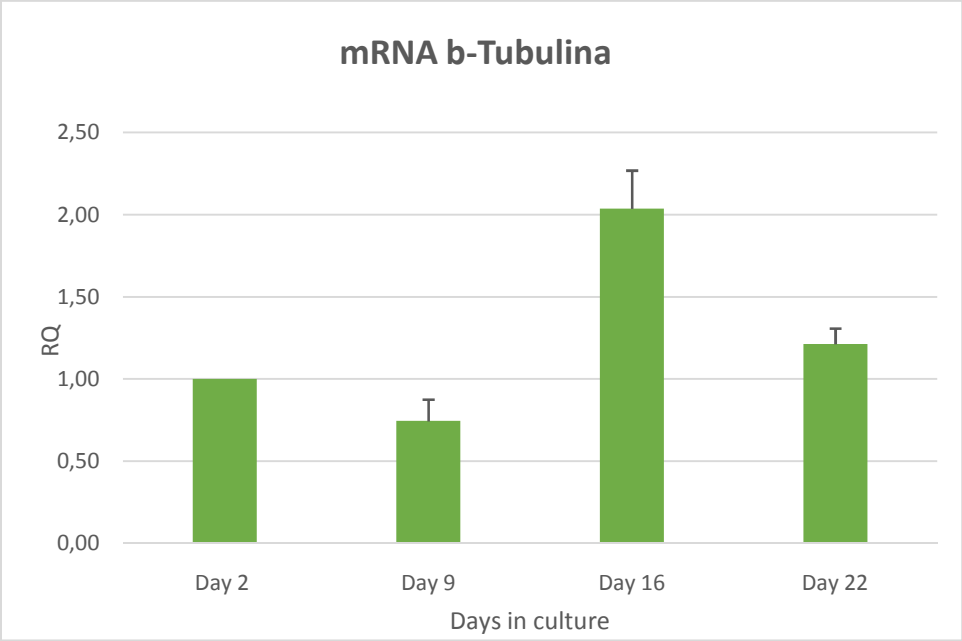


Fig.18 β- Tubulin mRna expression level at different day of Calu-3 culture.

Data are normalized to the housekeeping gene β Actin and quantified using the 2-ΔΔCt method.

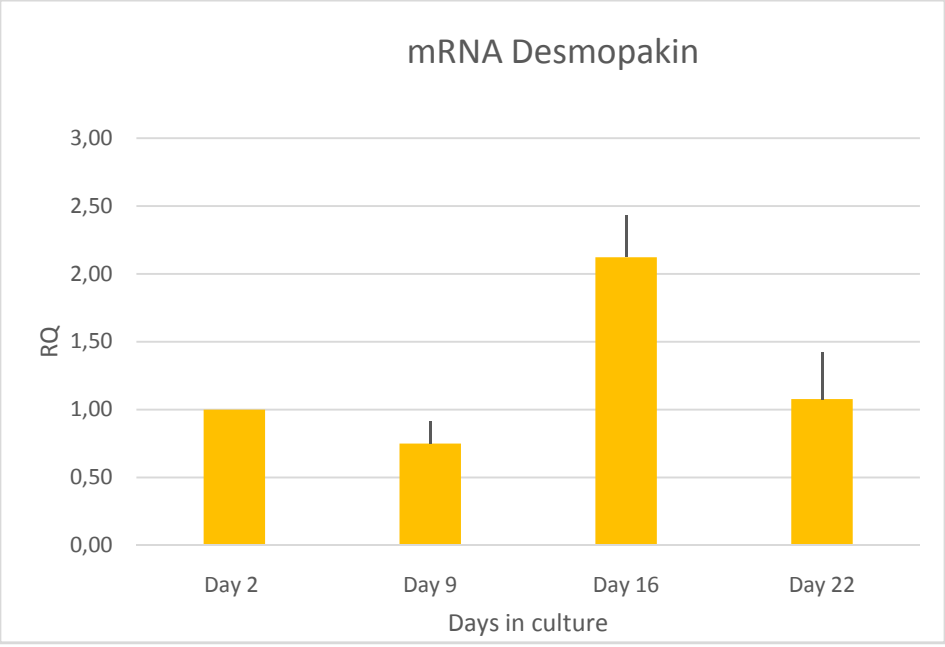


Fig 19DesmopakinmRna expression level at different day of Calu-3 culture.

Data are normalized to the housekeeping gene β Actin and quantified using the 2-ΔΔCt method.

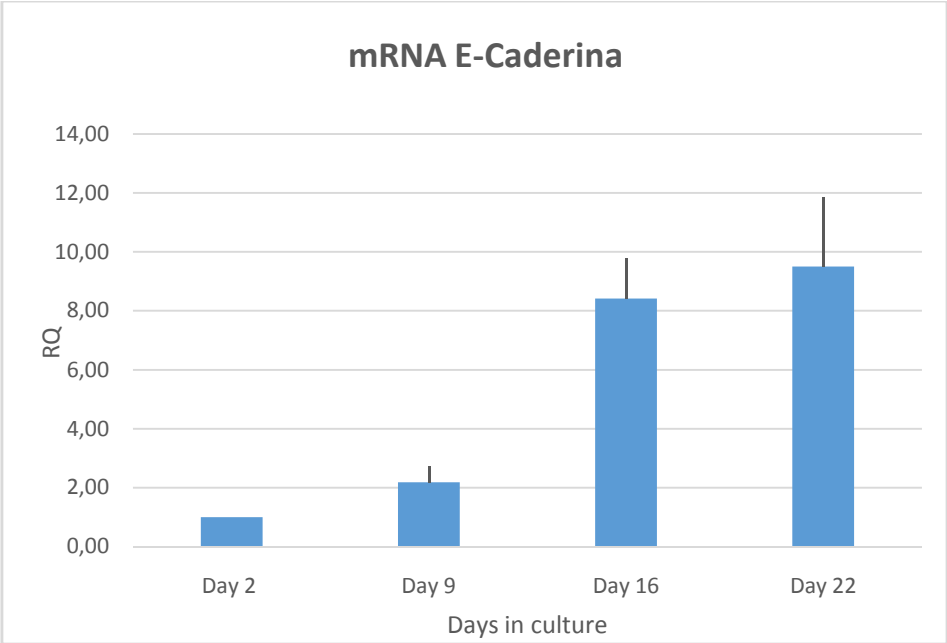


Fig.20E-CaderinmRna expression level at different day of Calu-3 culture.

Data are normalized to the housekeeping gene β Actin and quantified using the 2-ΔΔCt method.

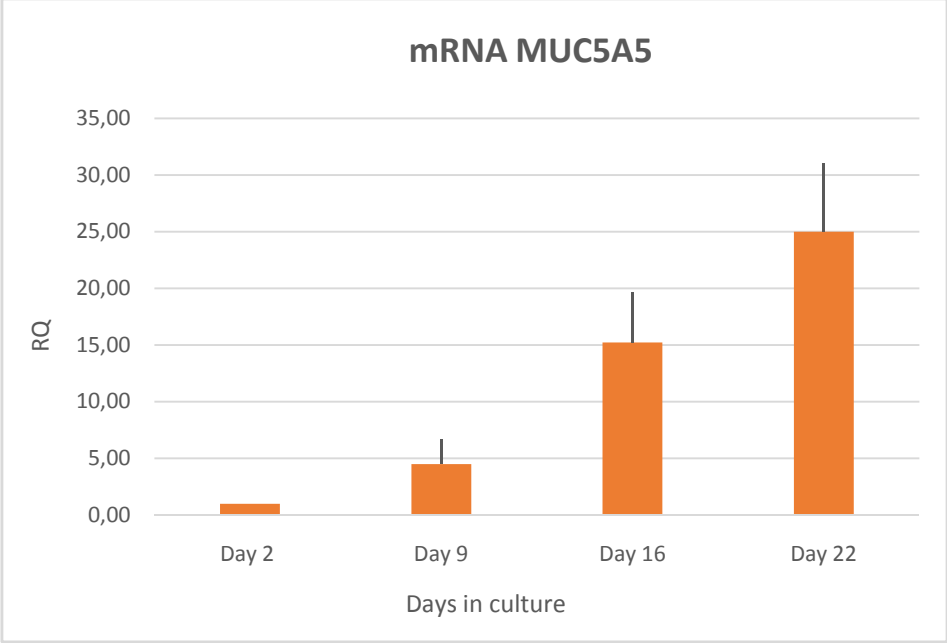


Fig 21MUC5AC mRna expression level at different day of Calu-3 culture.

Data are normalized to the housekeeping gene β Actin and quantified using the 2-ΔΔCt method.

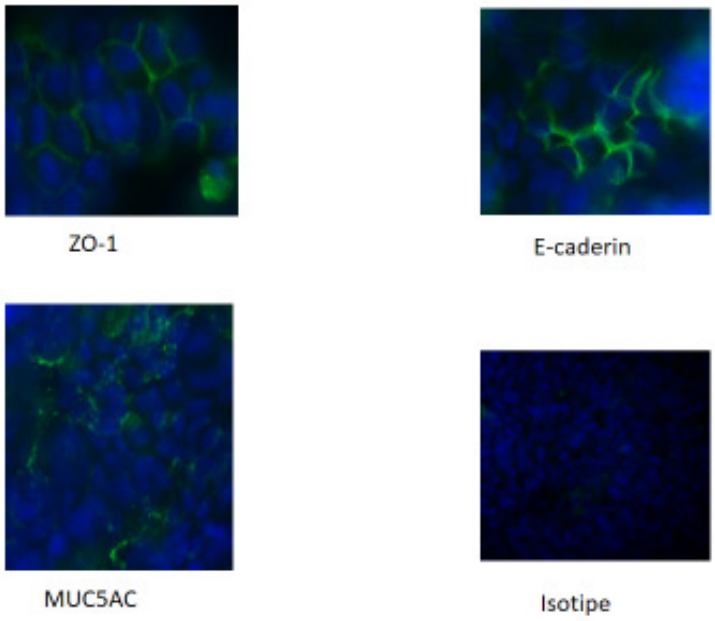


Fig 22. Representative images of immunostaininganalysis performed at day 16th of culture.

3.1.3 CALU-3 VALIDATION WITH REFERENCE COMPOUNDS

To validate the Calu-3 cell line as pulmonary permeability in vitro cell model, different reference compoundswere used: Propranolol and Metoprolol, known to have high epithelial permeability, Atenolol and Sulpiride, known to be low permeability compounds.

In addition, Digoxin, a known substrate of Pgp, was used to verify the presence and the function of this protein on the apical membrane.

As shown in Fig.23, Calu-3 monolayer was able to differentiate substances of low and high permeability. Papp of Propranolol e Metoprolol (1.1 and 1.4 * 10^{-5} cm/sec respectively) was found to be 2 log higher compared to Atenolol and Sulpiride (2.0 and 2.9 * 10^{-7} cm/sec respectively); in accordance with literature data, with Caco-2 cell model and in full agreement with in vivo absorption range.

It can also be observed that transport of Digoxin showed a directionality with higher B/A transport compared to A/B permeability (Fig. 24) confirming the presence and function of Pgp. In fact, the ER calculated for Digoxin was 9.3 (Fig.24), indicating an efflux transporter mechanism. The addition of both known Pgp inhibitors, Quinidine and Verapamil, led to an increase in permeability in A/B direction and, on the other hand, a decrease in B/A transport as results of Pgp activity inhibition. Also the reduction of ER when Quinidine and Verapamil were used, pointed out that the efflux protein involved in Digoxin transport is Pgp (Fig. 24).

These results confirm the presence and function of Pgp on apical membrane of Calu-3 cell line.

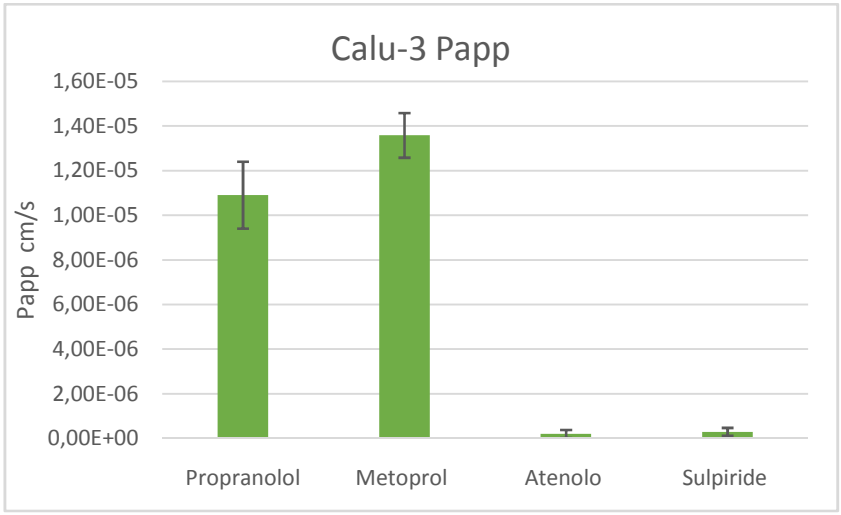


Fig. 23 Calu-3 Papp ofhigh and low permeability compounds.

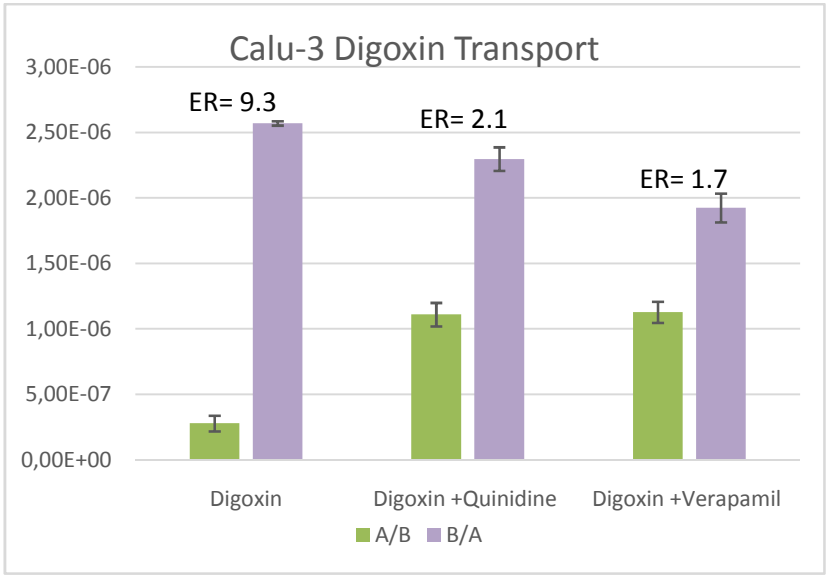


Fig. 24 Transport of Digoxin in A/B e B/A direction with and without Pgp inhibitors.

3.2 SIMULATED LUNG FLUID (SLF) SOLUBILITY ASSAY, LUNG TISSUE AND SLF BINDING ASSAY

A series of non-standard in vitro assays including lung tissue binding , SLF binding and SLF solubility assay, were carried out in order to supply more in vitro data, useful in understanding the different processes and aspects of pulmonary drug exposure.

In Table 2, the unbound drug percentage in lung tissue and in SLF is reported, revealing different affinity and binding properties among the chosen compounds.

In fact, the binding to proteins and phospholipids present in SLF, ranged from 20% to 98%, whereas the binding to lung tissue, structures in the cell membrane, receptors, cell organelles and tissue proteins varied from 96% to 99.9%

In Table 3, the SLF solubility of compounds is reported. The results showed large differences in solubility, ranging from 2000 µg/mL to 0.4 µg/mL.

COMPOUND	Lung Tissue % bound	SLF % bound
A	96.1	43.9
B	99.5	19.6
C	99.5	63.8
D	99.9	52.0
E	98.5	84.0
F	99.8	77.6
G	99.9	98.0
H	96.5	92.3

Tab. 2 Compound binding to lung tissue and simulated lung fluid.

COMPOUND	SLF solubility µg/mL
A	2425.96
B	2310.56
C	353.18
D	48.26
E	22.59
F	17.49
G	1.3
H	0.436

Tab. 3 Compounds solubility in simulated lung fluid.

3.3 CACO-2 AND CALU-3 Papp DETERMINATION

The permeability of a heterogeneous set of 8 locally acting drugs was assessed in the well-recognised model of gastrointestinal Caco-2 cell line and in the bronchial epithelial cellsCalu-3.
In tab. 4the apparent permeability coefficients estimated in the two model were reported.
As highlighted from the data, the Papps were quite different between the two cells systems.
While in Caco-2 cell line the compounds showed high, medium and low permeability features, in Calu-3 cell model the majority of compounds revealed very low permeability properties and only one compound , compound D, exhibited absorption characteristic in the medium range.
Comparison with other non-pulmonary cell types like Caco-2 gastrointestinal model, showed that lung epithelium has different properties that could be better mimed from in vitro model from airway tract.

	Papp 10^{-7} cm/sec	
COMPOUND	**Calu-3**	**Caco-2**
A	1.03	12.02
B	0.99	30.40
C	0.27	17.70
D	46.40	105.00
E	0.75	9.60
F	0.47	81.70
G	0.06	3.10
H	0.37	3.06

Tab.4 Papp estimation in Caco-2 and Calu-3 cell lines.

3.4 IN VITRO IN VIVO CORRELATION

For the IVIV correlation, the apparent permeability coefficients estimated both in Caco-2 and Calu-3 cells line were correlated with different pharmacokinetic parameters that describe the drug disposition in lung: Lung drug half-life (T ½), Lung mean residence time (MRT), Mean absorption time (MAT) and the percentage of drug retained in the lung at 24h after dose.
Papp valueswere correlated with each of these in vivo PK parameters for the establishment of an In vitro-In vivo correlation.

3.4.1 CACO-2 IVIVC

When Papp from Caco-2 were considered, the analysis of the data showed a good correlation between the logarithm (log) of the rat lung T ½ plotted against the log of Caco-2 Papp, resulting in a log linear correlation with a correlation coefficient $r^2 = 0{,}91$ (Fig.25).
A support of this results, also when considering the lung MRT parameter a regression with $r^2 = 0$, 95 could be established (Fig.26).

Regarding the Caco-2 Papp and the % of lung retention at 24h, a polynomial correlationwith a r^2 = 0,98 (Fig.27) well described the inter-parameter relation as at higher in vitro permeability value correspond a low % of drug retained by the lung (and so an high systemically absorption).

On the contrary, when the Papp value decrease there is an exponential increase in the percentage of drug found in the lung. This finding could give precious information in understanding the difficult balance between lung tissue retention and permeability addressed when a drug is designed for pulmonary delivering.

Considering the MAT parameter, a linear correlation was found only for 6 of the 8 compounds tested (Fig.28), furthermore these 6 compounds were all located in the far-end of the regression line. No-compounds were found in the centre part of correlation, thus distrusting the reliability of correlation. An explanation of this finding could reside in the difficulty on an accurate estimation of MAT, compared to others PK parameters considered.

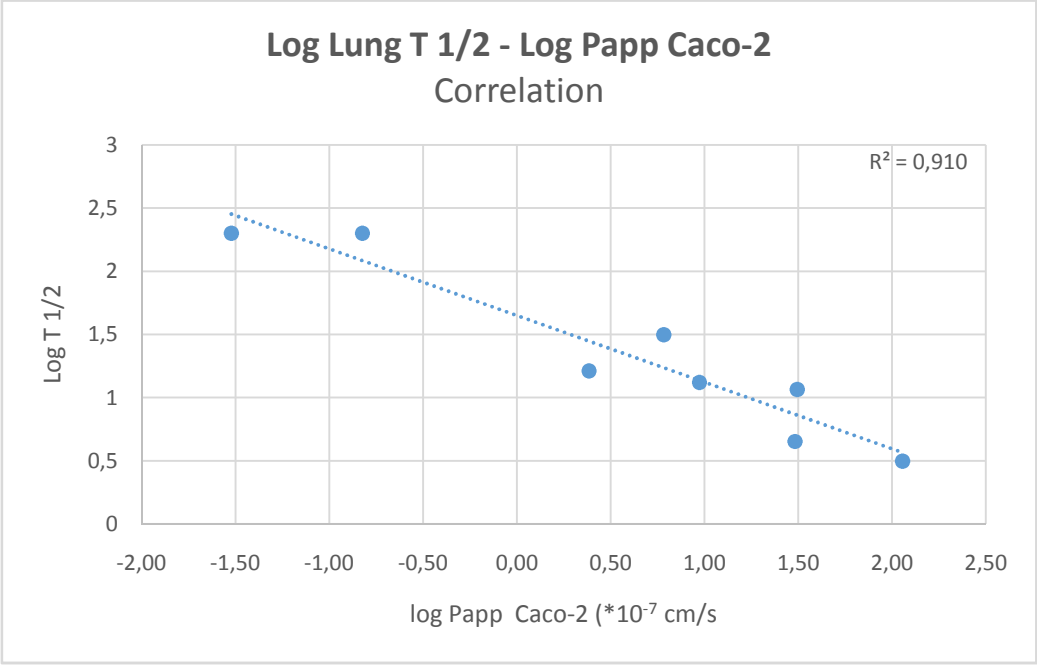


Fig.25 Correlation of Caco-2 permeability with in vivo rat lung T½ .

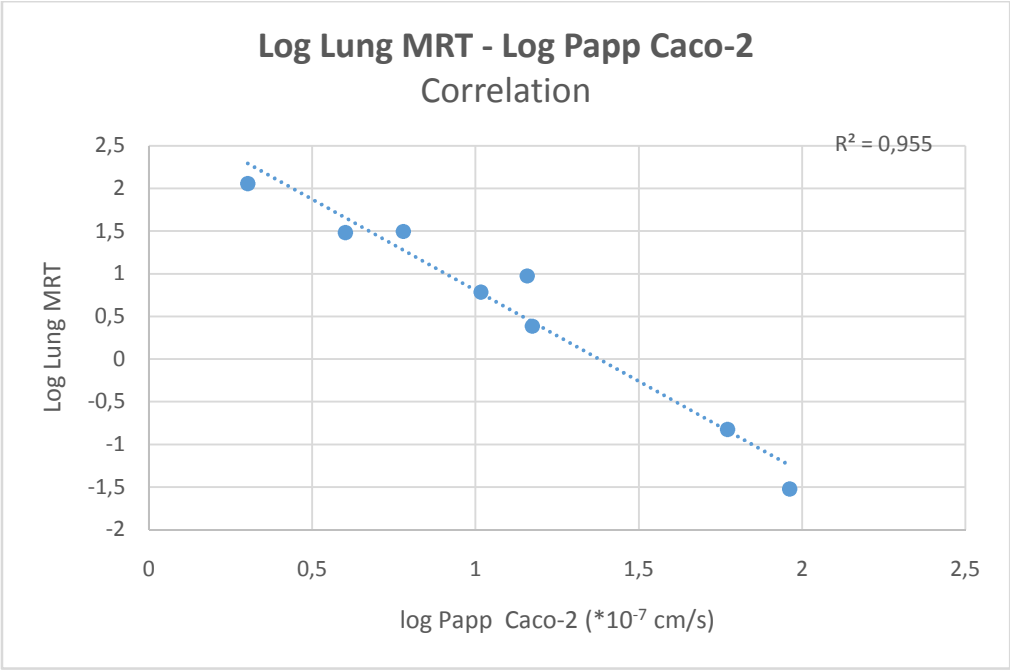


Fig.26 Correlation of Caco-2 permeability with in vivo rat lung MRT.

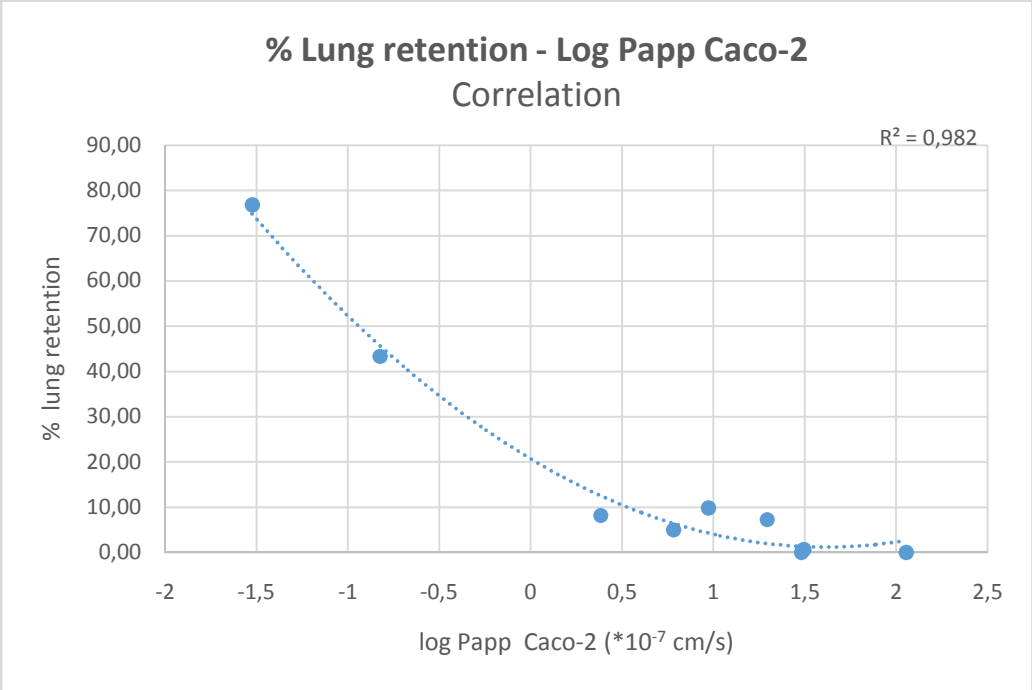


Fig 27 Correlation of Caco-2 permeability with percentage of in vivo rat lung retention.

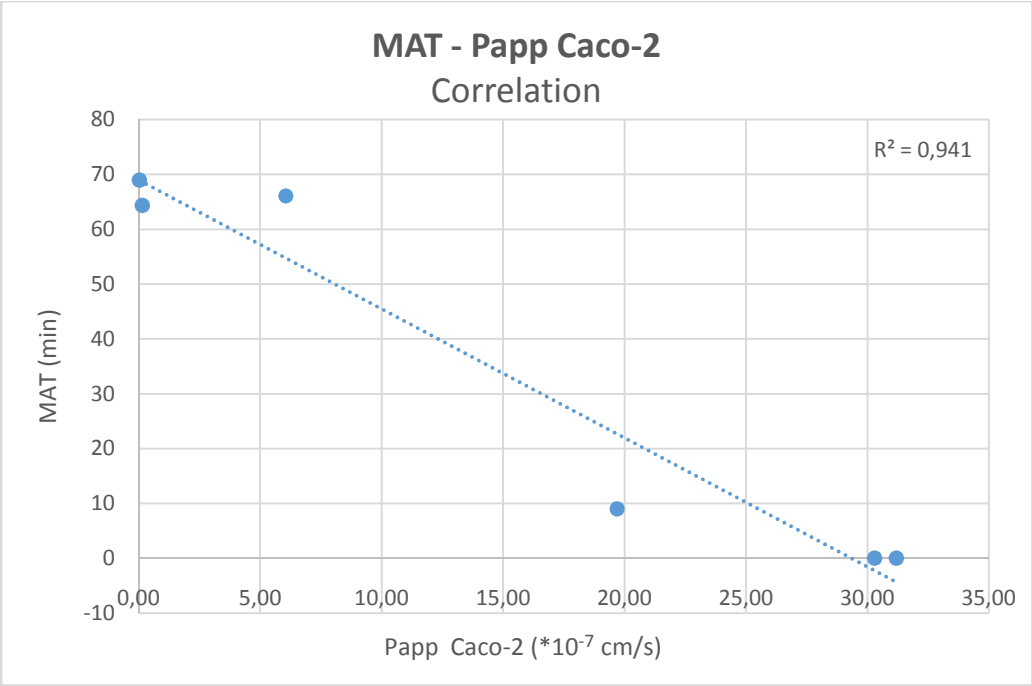


Fig. 28 Correlation of Caco-2 permeability with in vivo MAT.

3.4.2 CALU-3 IVIVC

The same set of compounds was tested also in Calu-3 cells model for permeability evaluation and the respective Papps were put in relation with the selected in vivo PK parameters to find out if the In Vitro-In Vivo correlationsobtained with Caco-2 cells, were confirmed, improved or rejected.

As for Caco-2 model, also Calu-3 Papp well correlated with the in vivo data considered.

In particular, a linear correlation with a correlation coefficient r^2 = 0, 8 was found when the logarithm (log) of Calu-3 Papp was plotted against the log of the rat lung T ½ (Fig.29).

In a similar manner, the correlation between in vitro permeability value and in vivo lung MRT parameters was confirmed also with Calu-3 cells line (Fig.30).

Moreover, the relationship between in vitro permeability and the PK parameter of lung retention was well described by the exponential correlation of log Calu-3 Papp plotted against the log % of lung retention at 24h, resulting in a r^2 = 0,9 (Fig.31).

On the contrary, a poor correlation was found when MAT parameter was considered (Fig.32), confirming that this parameter is not suitable for the establishment of IVIV correlation, probable because its accurate and precise measure continue to be a challenge.

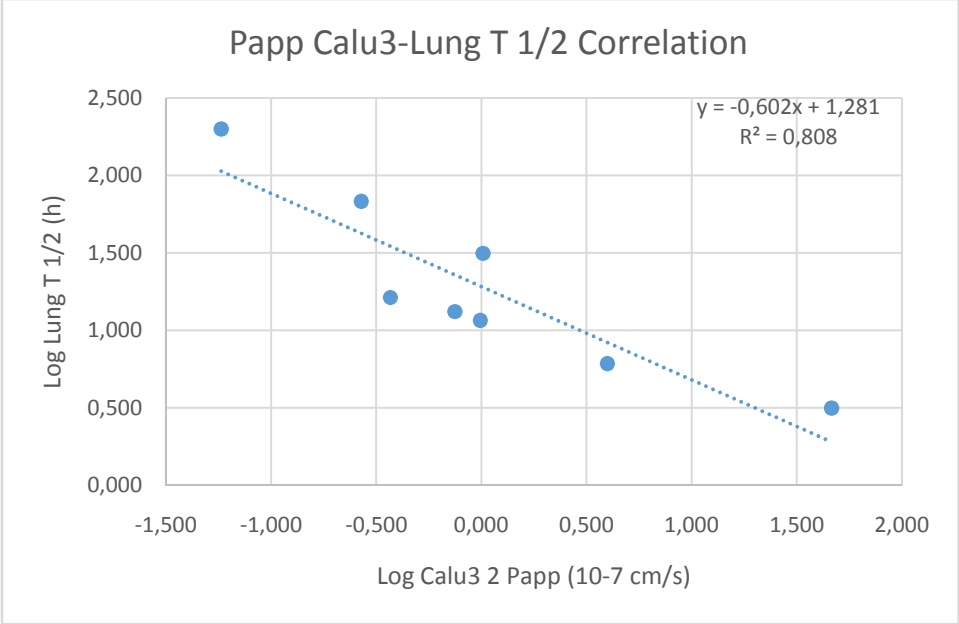


Fig.29Correlation of Calu-3 permeability with in vivo rat lung T1/2.

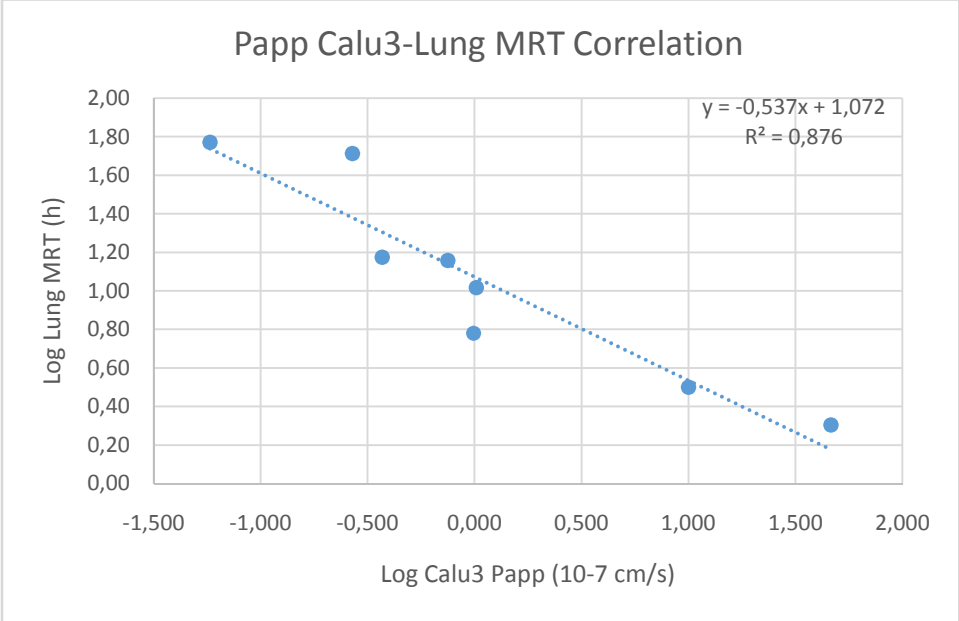


Fig. 30Correlation of Calu-3 permeability with in vivo rat lung MRT.

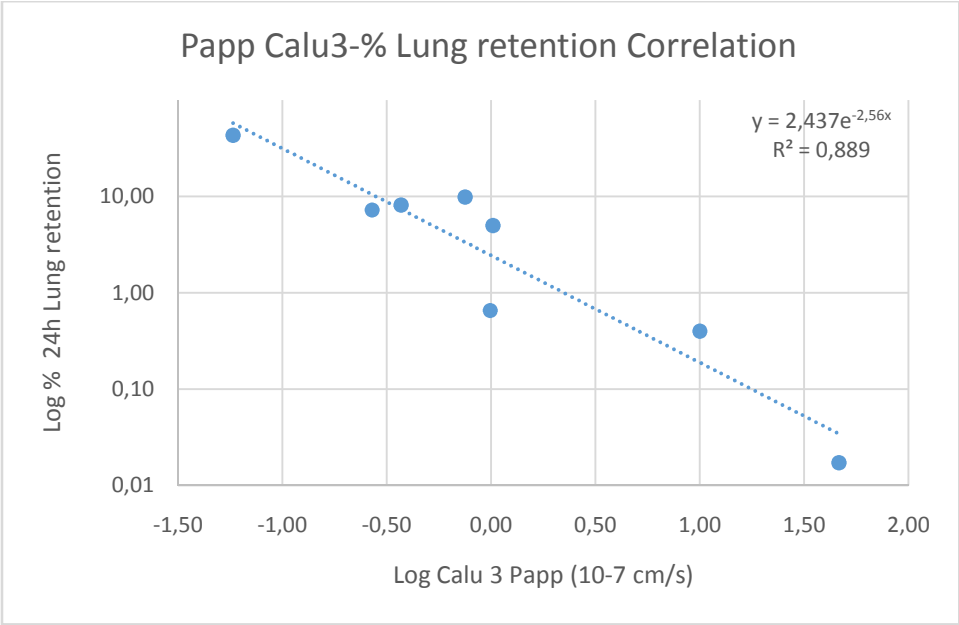


Fig. 31Correlation of Calu-3 permeability with percentage of in vivo rat lung retention.

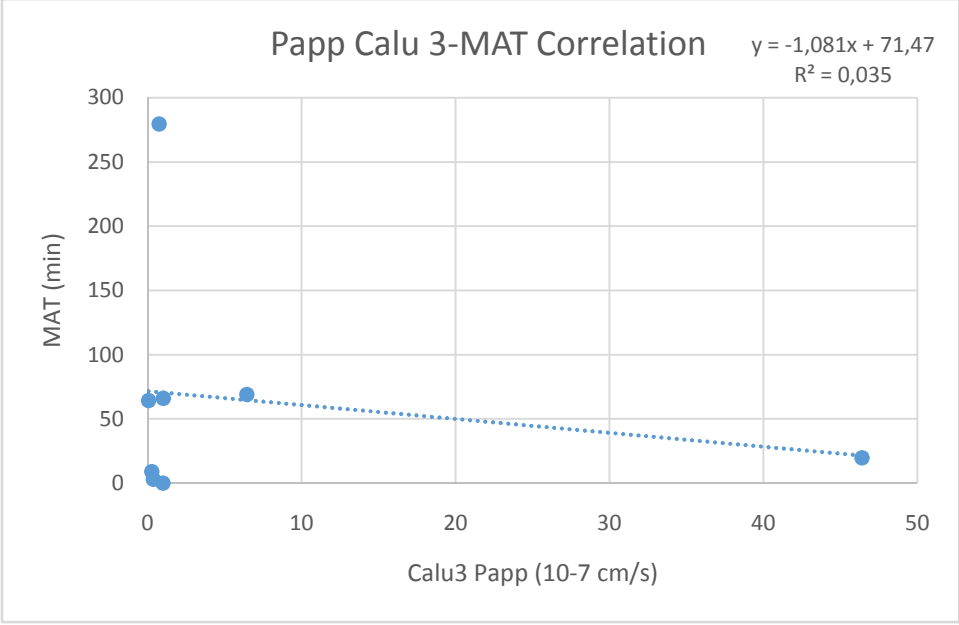


Fig. 32Correlation of Calu-3 permeability with in vivo MAT.

3.5 PBPK SIMULATION

All generated in vitro data were included in a PBPK model built on rat physiological parameters to simulate the concentration-time profile of drugs in plasma and lung after IT administration.
The simulations were then comparedwith the actual in vivo data in rats.
In Fig. 33e 34, the simulated and observed PK profiles in plasma and lung after IT administration of compound A are shown. As in vitro permeability, both Caco-2 (Fig. 33) and Calu-3 Papp (Fig.34) values were used to find out if a better simulation was achieved with one of the two cell lines.
As illustrated by the graphs, the concentration-time curves generated starting from Calu-3 Papp value were more close the actual PK profile both in plasma and in lung. The lower Papp estimated in Calu-3 cells ($1.03 *10^{-7}$ cm/sec)carried to a curve with a less steep slope compared to Caco-2 simulation. This evidence was more pronounced in lung kinetic where the high value of Caco-2 cells ($12.02 * 10^{-7}$ cm/sec) led to a simulation with a very steep curve where the terminal part of compound kinetic was underestimated.
A support of this finding, also the estimation of lung PK parameters was better achieved with Calu-3 cells simulation (Tab.5).
The key parameters here considered are lung Tmax, Cmax, AUC, MRT and T ½ .
Briefly, Cmax is the maximum concentration of drug found in lung after dosing.The related pharmacokinetic parameter Tmax is the time at which the Cmaxis observed. AUC means area under the exposure-time curve, indicates total lung exposure to drug, MRT, is the average time that the drug stays in the lung prior to elimination and T ½ is the time taken for half of the dose to be eliminated.
In Tab. 5, the ratio between observed versus simulated lung parameters is reported from both Caco-2 and Calu-3 simulation. A value close the unity indicates a very good simulation, a value > 1 denotes an underestimation; a value <1 reveals an over prediction.
As shown in Tab. 5, Cmax and Tmax were well estimated from both Caco-2 and Calu-3 cell model, whereas AUC, MRT and T ½ were better predicted from Calu-3 cells as the observed/simulates ratios were close the unity.
A similar finding was found for all other compounds analysed whom lung disposition was better simulated when Calu-3 Papp was used.
In Figures from 35 to 39, the lung profiles and PK parameters of compounds C, D, E, G and Hare reported.
For compound B and F, PBPK modeling exploration is still on-going.

As shown in the graphs below, also in these cases, the drug profile in lung after dosing was better described from Calu-3 simulation. For some compounds, like compound C, and D (Fig. 35,36) the comparison between the two simulation revealed a certain improvement of prediction when Calu-3 cells model was used since the green line of Calu-3 simulation was always more proximal to the blue line of observed lung profile if compared with the red line of Caco-2 simulation profile.

For others compounds, like E, G and H (Fig.37, 38, 39), the simulation was only achieved with Calu-3 cells model as long as the concentration-time curve simulated from Caco-2 was very steep and very far compared with the actual lung profile.

In the same way, also the estimation of lung PK parameters for compound A,C and D was certainly improved with Calu-3 simulation because the ratio is always more proximal to unity compared with Caco-2 simulation . But for compounds E, G and H, this finding was even more pronounced since the ratio observed/simulated of Caco-2 cell model ranged from 1.7 to 8.74 for AUC, MRT and T ½ parameters showing a big difference between the predicted and actual value. Whereas with Calu-3 simulation this ratio remained between 1 and 2.2 revealing a powerful prediction tool. (Fig. 35-36-37-38-39)

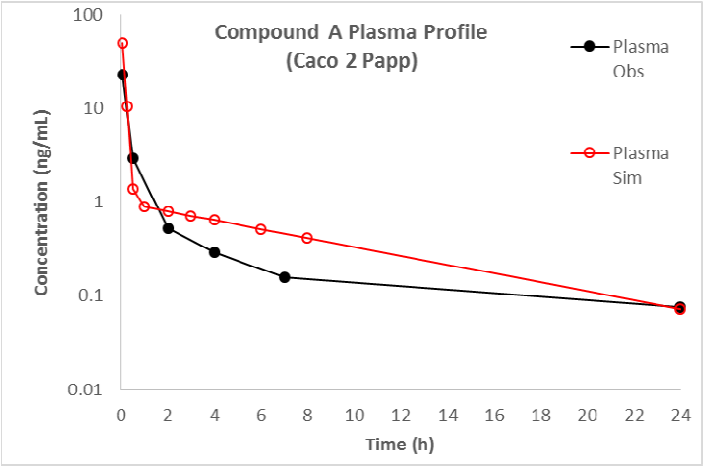
Given these promising prediction of lung kinetic with Calu-3 cell model, also the simulation of concentration-time profile in plasma was investigated.

In fig. 40 to 44 the simulated and observed PK profiles in plasma after IT administration are shown.

For the majority of the compounds (compound A, C, D, E, G) the kinetic in plasma was well simulated from PBPK modelling with the exception of compound H that revealed a different profile in plasma.

For compounds B and F, PBPK modeling exploration is still on-goingbut these first results related to other compounds, suggests that Calu-3 permeability estimation could be more predictive of drug lung absorption

Overall the results showed that Calu-3 cells line used in a PBPK modelling gave the right and proper input parameters to better describe the absorption process of pulmonary drug, leading for some compounds, to an improvement in prediction in comparison with Caco-2 and even better, for other compounds, making prediction possible.



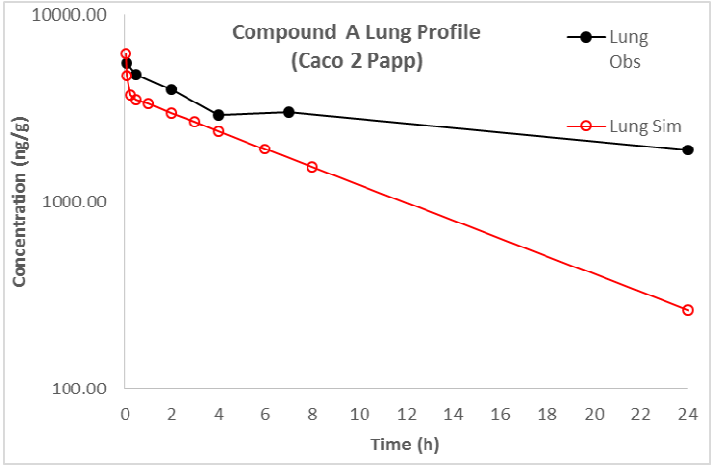
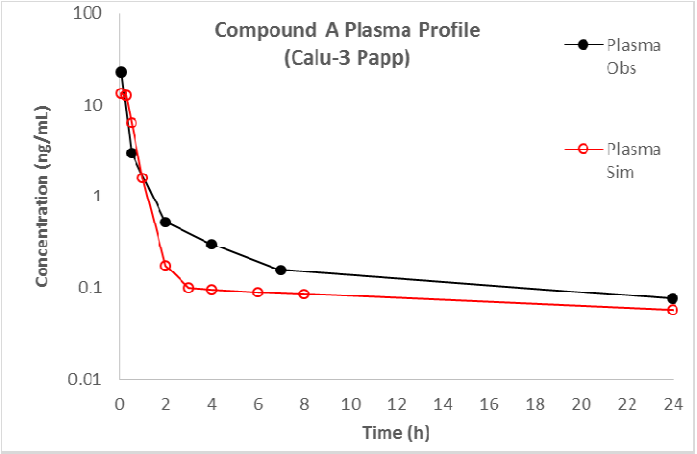


Fig. 33 PBPK modeling for compound A using Caco 2 Papp (12.02 *10 $^{-7}$cm/sec)



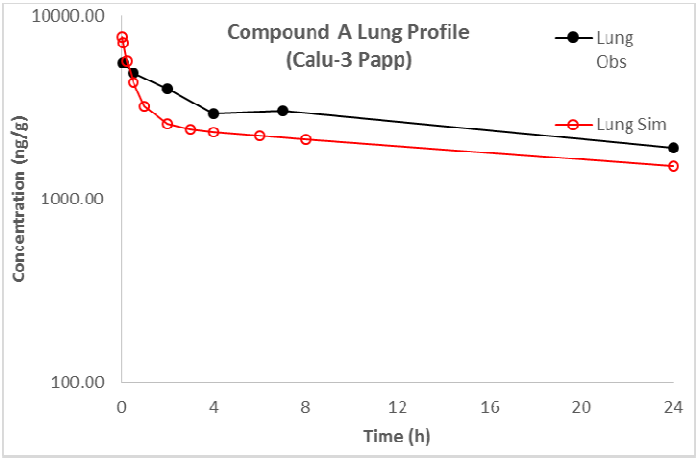
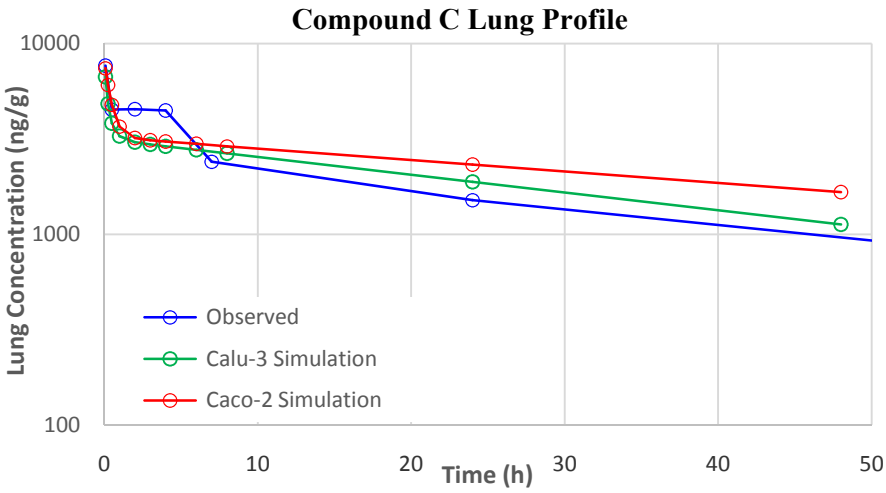


Fig. 34PBPK modeling for compound A using Calu-3 Papp (1.03 *10 $^{-7}$cm/sec)

OBSERVED/SIMULATION RATIO

	Tmax	Cmax	AUC	MRT	T ½
Caco-2	1.00	0.94	2.18	2.13	2.18
Calu-3	1.00	0.93	1.18	0.92	0.94

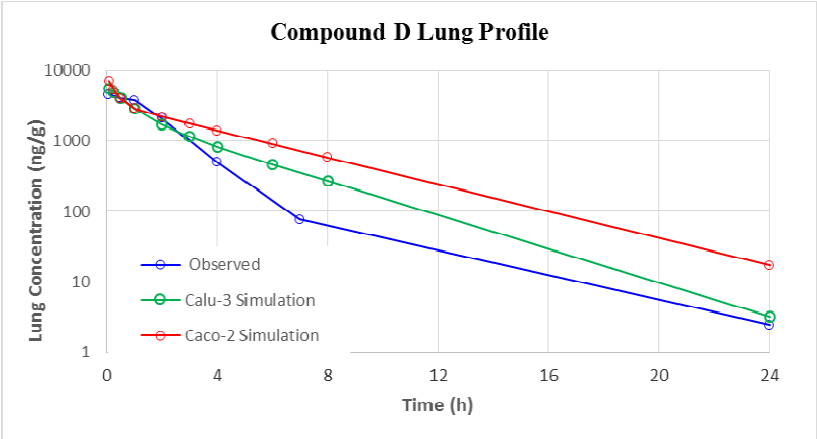
Tab.5 Observed/Simulated Ratio of lung PK parameters of Compound A



OBSERVED/SIMULATION RATIO

	Tmax	Cmax	AUC	MRT	T½
Caco-2	1.00	1.03	0.59	0.64	0.69
Calu-3	1.00	1.14	0.95	1.00	1.07

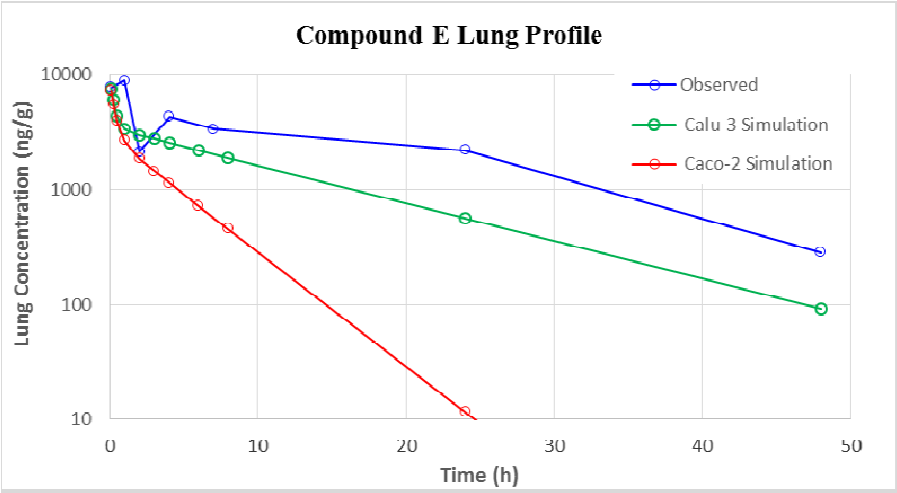
Fig. 35 Lung profile and PK parameters ofcompound C



OBSERVED/SIMULATION RATIO

	Tmax	Cmax	AUC	MRT	T ½
Caco-2	1.00	0.64	0.60	0.47	1.04
Calu-3	1.00	0.81	0.87	0.67	1.29

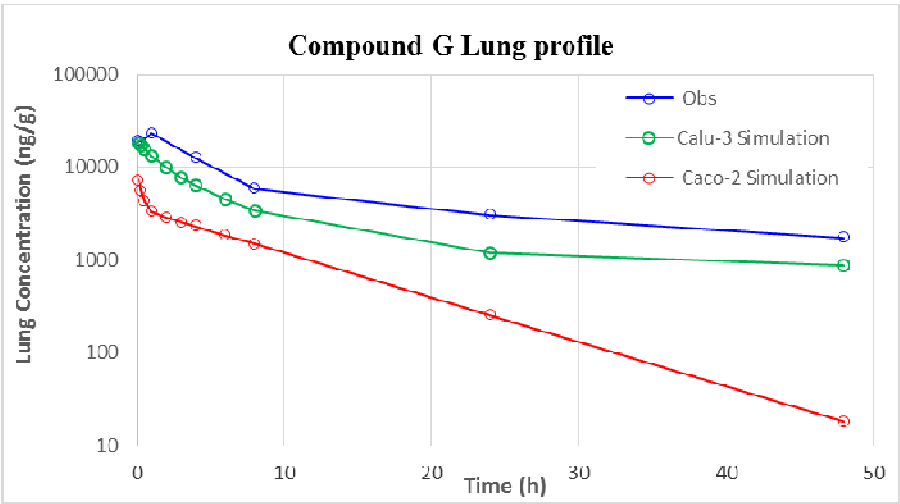
Fig. 36 Lung profile and PK parameters of compound D



OBSERVED/SIMULATION RATIO

	Tmax	Cmax	AUC	MRT	T ½
Caco-2	12.50	1.24	7.27	4.45	3.50
Calu-3	12.50	1.18	2.20	1.34	1.16

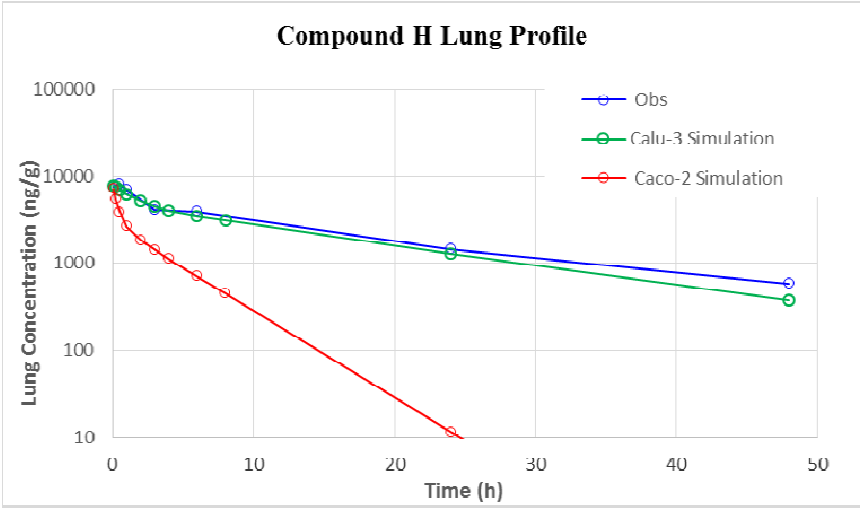
Fig. 37Lung profile and PK parameters of compound E



OBSERVED/SIMULATION RATIO

	Tmax	Cmax	AUC	MRT	T 1/2
Caco-2	12.50	3.17	8.74	1.69	3.90
Calu-3	12.50	1.28	1.98	1.11	1.09

Fig. 38 Lung profile and PK parameters of compound G



OBSERVED/SIMULATION RATIO

	Tmax	Cmax	AUCinf	MRTinf	T ½
Caco-2	6.25	1.14	7.85	5.63	5.31
Calu-3	6.25	1.04	1.19	1.20	1.21

Fig. 39 Lung profile and PK parameters of compound H

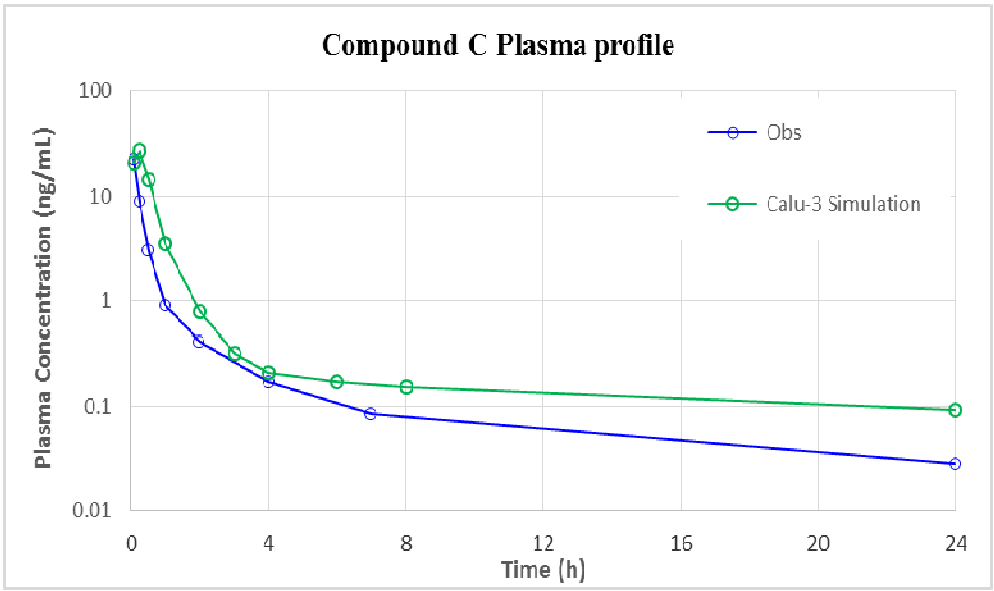


Fig. 40 Plasma profile of compound C

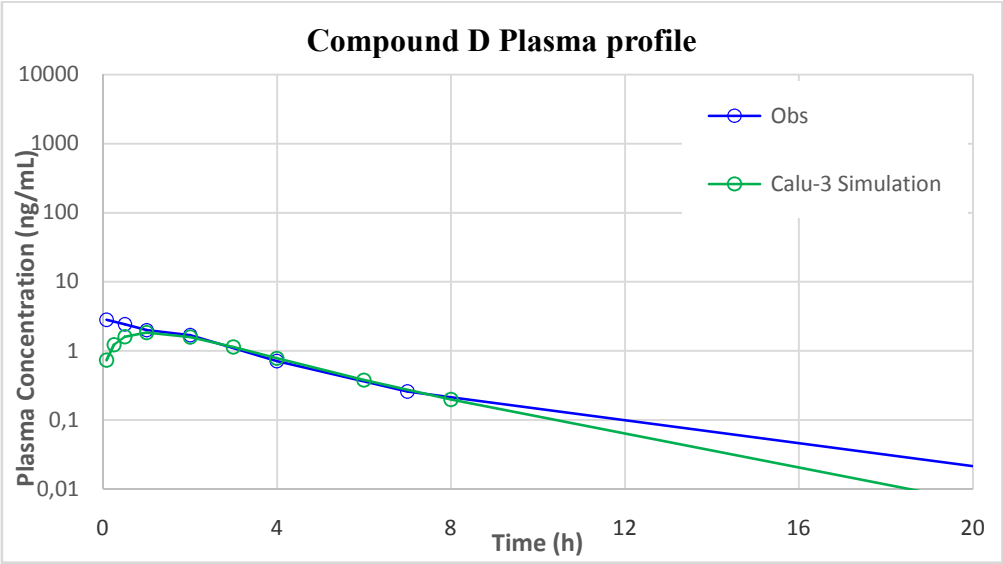


Fig.41 Plasma profile of compound D

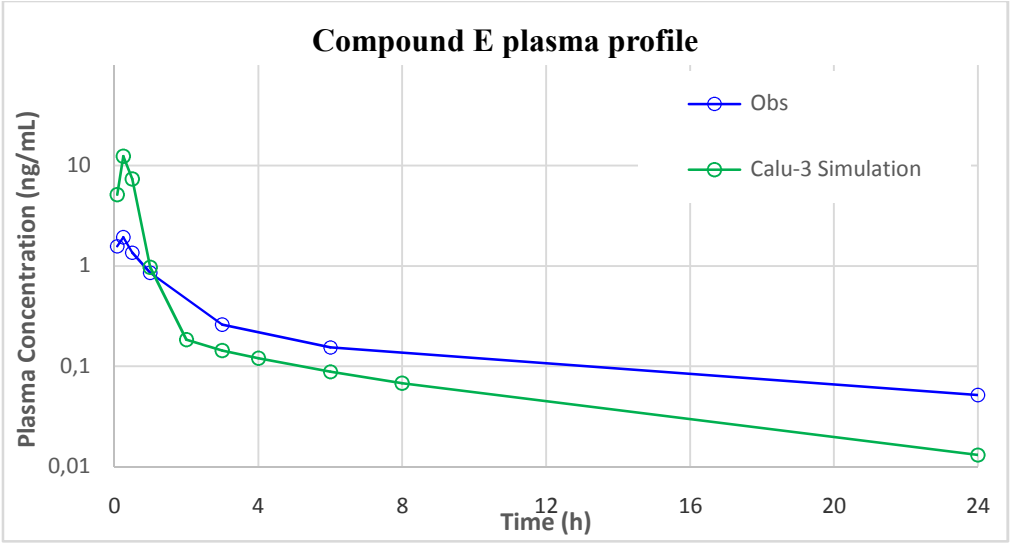


Fig.42 Plasma profile of compound E

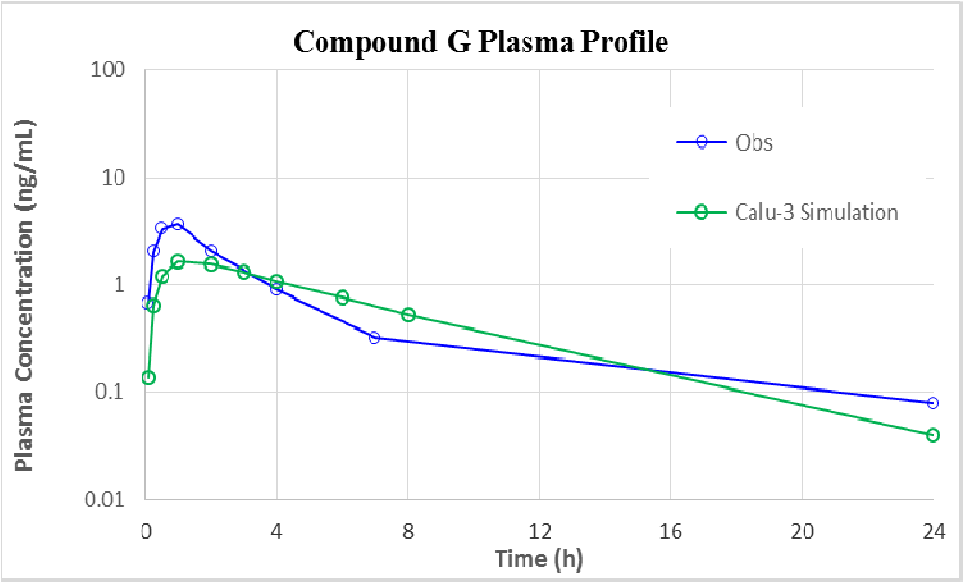


Fig.43 Plasma profile of compound G

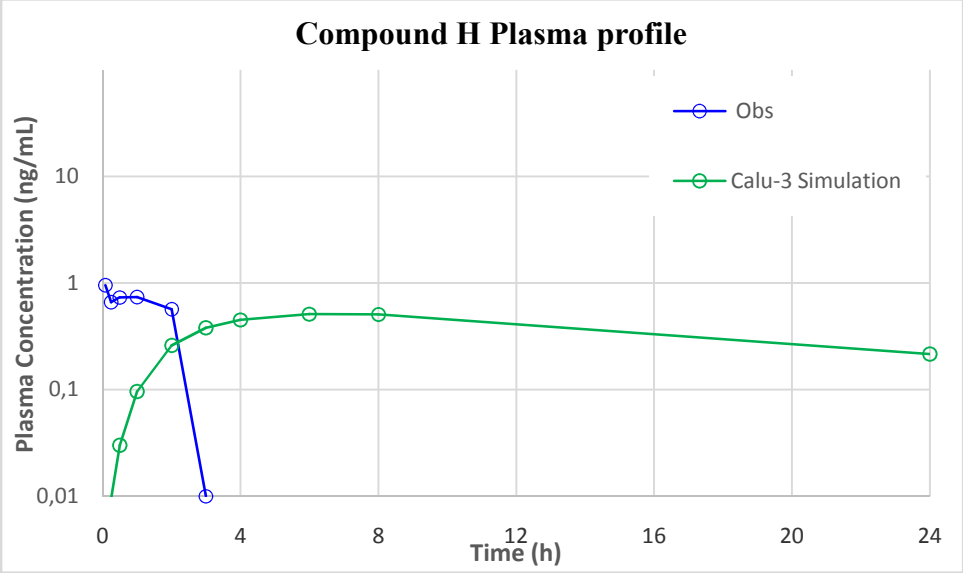


Fig.44 Plasma profile of compound H

CHAPTER IV

4. DISCUSSION

Within the past few decades, the lung has received increasing attention as a target for local and systemic drug delivery (1, 2).

If for treatment of respiratory disease, the benefits of pulmonary drug delivery are well known and have been appreciated since years, inhalation of medicinal aerosol to the lung for deliveryinto systemic circulation has developed recently into one of the most promising alternatives to oral or invasive routes of administration.

Drug administration via the respiratory tract appears promising because of the peculiar anatomical and physiological characteristics of the lung: large surface area, thin alveolar epithelium, high vascularization and relatively low level of metabolic enzymatic activity(3, 4).

However, the development of safe and effective medicines for administration via the lung will require not only the optimisation of device and formulation, but also an understanding of respiratory biopharmaceutics and the interaction of inhaled medicines with lung (2).

While the technology for pulmonary drug delivering has improved, the understanding of how inhaled medicines interact with the lung remains basic. Thus, sophisticated formulations are being developed and drugs are being designed with the aim of avoiding or promoting interaction with lung but methods for evaluating or optimising these delivery strategies are lacking (2).

Therefore, the recent developments in delivering drugs to the lung are driving the need for in vitro methods to evaluate the fate of inhaled medicines. In vitro techniques to evaluate drug and formulation interaction with the lung, drug binding, uptake, transport (including rate, mechanism and extent of absorption) and metabolism in the lungare required.

The use of epithelial cell culture in research and development to investigate many of these aspects is well established since the acceptance of cell culture methods by the pharmaceutical industry has been facilitated by the predictive ability and the higher and quicker throughput offered by these systems compared to in vivo tests employing animals (8).

In the oral delivery arena, in vitro cell culture model of gastrointestinal Caco-2 cell line have been usedextensively to screen the oral absorption potential of drugs and their mechanism of permeation across the epithelial barrier so much that it is now considered the gold standard system for absorption studies (20).

In pulmonary drug delivery, however, there is no lung-equivalent of Caco-2 cells to serve as well-established and reliable in vitro model of the respiratory epithelium (9, 10).

Recently, the continuously growing human bronchial epithelial cells, Calu3 was identified as promising candidate as drug absorption model of airways. This cell line exhibits many of the features of primary cells displaying a polarized well-differentiated epithelium with essential drug transporter proteins and functional tight-junction.

Therefore, the goal of this study was to evaluate the potential of Calu-3 cells as a screening tool to predict the rate and extent of in vivo lung absorption.

For this purpose, the barrier properties and cells differentiations state of Calu-3 linewere investigated to provethe similarity with the native airway epithelium.

First, the capacity to form tight junction was evaluated through the measure of the transepithelial electrical resistence. The data showed that Calu-3, grown under AIC condition, developed resistance as function of time reaching a TEER > 300 ohm-cm2 from day 16 up to day 35 of culture. At the same time, the permeability estimation of paracellular marker Fluorescein Sodium Salt exhibited a significant decrease in permeability of Fluorescein from day 2 to day 35 reaching the expected Papp value $< 2*10^{-6}$ on day 16 . This is consistent with the TEER measurement results described above.

In addition to this, the expression of differentiation and cell adhesion markers MUC5AC (globlet cells), β-Tubulin IV (ciliated epithelial cells), ZO-1 (tight junction protein), E-Cadherin and Desmopakine (cell adhesion molecules and epithelial cell markers) was evaluated through immunostaining and quantitative polymerase chain reaction (qPCR).

qPCR analysis revealed a time dependent expression of the selected markers in Calu-3 cell model, an expression that increased with the days in culture, from day two to day 22. Alsoimmunostaing, performed at day 16, showed an high presence of these protein in the cells culture.

Moreover, transport studies with different reference compounds, showed that Calu-3 monolayer was able to differentiate substances of low and high permeability characteristics. Papp of Propranolol e Metoprolol (high permeability) was found to be 2 log higher compared to Atenolol and Sulpiride (low permeability); in accordance with literature data, with Caco-2 cell model and in full agreement with in vivo absorption range.

It can also be observed that transport of Digoxin, a known substrate of Pgp (the major transporter involved in xenobiotic cell efflux), showed a directionality with higher transport in basolateral to apical direction compared to apical-basolateral permeability confirming the presence and function of this important efflux transporter in human airway epithelium.

Overall, these data demonstrate that Calu-3 exhibit many of the features of primary cells forming a uniform layer of polarized, well-differentiated cells with mucus secretion, functional tight-junction and important transporter proteins confirming its potential use as a model to assess lung permeability.

Once set up and validated, Calu-3 cells model was used to assess the in vitro Papp of a set of 8 locally acting drugs of varying molecular size, charge, lipophilicity, and polar surface area.

These compounds were then tested also in the in the well-recognized Caco-2 cells model, considered the gold standard system for absorption studies.

Commonly, permeability data from cell culture, offers a convenient reproducible and quantifiable way to evaluate the absorption potential of a compound, leading to a rank order that highlights qualitatively those compounds that show favourable permeability characteristics.

In the present study, it was demonstrated how Calu-3 and Caco-2 permeability data can be applied to quantitatively predict the extent of absorption in vivo.

In fact, the apparent permeability coefficients estimated both in Calu-3 and Caco-2 cells line were compared to in vivo absorption data in rats to find out if a correlation could be established.

For the IVIV correlation, different pharmacokinetic parameters have been considered: Lung drug half-life (T ½), Lung mean residence time (MRT), Percentage of drug retained in the lung at 24h after dosing, Mean absorption time (MAT).

By plotting the logarithm of Calu-3 or Caco-2 Papp with the log of rat lung T ½ , the two rate parameters were mathematically correlated to establish a relationship that capture the same event in drug absorption process.

Also when considering the lung MRT parameter and the percentage of drug retained in the lung at 24h, a regression with a good r^2 could be established with both Caco-2 and Calu-3 cells lines.

On the contrary, a poor correlation was found when MAT parameter was compared with Calu-3 Papp as well as with Caco-2 values. An explanation of this finding could reside in the difficulty on an accurate estimation of MAT compared to others PK parameters considered, revealing that this parameter is not suitable for the establishment of IVIV correlation.

Overall these results obtained with Caco-2 and Calu-3 cell line, highlighted the inter-parameter relation found between in vitro permeability data and three in vivo PK parameters: lung T ½ , lung MRT and % of drug retained in the lung at 24h after dose, leading to establishment of a strong and reliable IVIV correlation.

In future, in vitro permeability data couldbe used to guide drug discovery programs allowing prediction and estimation of lung kinetic of inhaled drug.

To deep analyse the predictive power of Caco-2 and Calu-3 cell lines for permeability studies in pulmonary drug delivery, the two cells model were evaluated and compared to each other through physiologically-based pharmacokinetic (PBPK) modelling.

First, for all selected compounds, routine ADME assays were performed (Clearance in hepatocytes and microsomes, plasma protein binding).

After that, a series of non-standard in vitro assays including lung tissue binding (LTB), simulated lung fluid (SLF) binding and SLF solubility assay, were carried out in order to supply more in vitro data, useful in understanding the different processes and aspects of pulmonary drug exposure.

The results revealed different affinity and binding properties to SLF and LTB among the chosen compounds as well as showed large differences in solubility, ranging from 2000 µg/mL to 0.4 µg/mL.

All generated in vitro data were included in a PBPK model built on rat physiological parameters to simulate the drug concentration-time profile in plasma and lung after IT administration.

The simulations were then comparedwith the actual in vivo data in rats.

For all the compounds analysed, the concentration-time curves in lung generated starting from Calu-3 Papp value were more close the actual PK profile. The lower Papp estimated in Calu-3 cells carried to a curve with a less steep slope compared to Caco-2 simulation that led to a simulation with a very sloped curve where the terminal part of compound kinetic was alwaysmiscalculated.

In particular, for some compounds, like compound A, C, and D, the comparison between the two simulation revealed a certain improvement of prediction when Calu-3 cells model was used, but for others compounds, like E, G and H the simulation was only achieved with Calu-3 cells model.

In the same way, also the estimation of key lung PK parameters was better achieved with Calu-3, since the ratio between the observed versus simulated parameter is often more proximal to unity in comparison to Caco-2 simulation .

For compounds B and F, PBPK modelling exploration is still on-going but these first results related to other compounds, suggests that Calu-3 permeability estimation could be more predictive of drug lung absorption

Overall the results showed that Calu-3 cells line used in a PBPK modelling gave the right and proper input parameters to better describe the absorption process of pulmonary drug, leading for some compounds, to an improvement in prediction in comparison with Caco-2 and even better, for other compounds, making predictionpossible.

Comparison with other non-pulmonary cell types like Caco-2 gastrointestinal model, showed that lung epithelium has different properties, i.e it is less permeable.

Fundamentally, the role of the gut is to accept foreign material for processing, uptake and expulsion. In contrast, the lungs are designed to remove foreign material in order to maintain gas exchange that necessitated evolution of a variety of effective clearance mechanisms (15). At a cellular level the general mechanism of uptake and absorption are probably quite similar to those understood for the gut, in fact predictive relationships are not exclusively obtained by the airway cell cultures Calu-3 but may be obtained also using the non-respiratory epithelial cell Caco2. However, at the macro level the upper airway of the lungs are quite different to the gastrointestinal barrier, having a thick mucus layer and high level of defence mechanisms (15, 36, 37).

In future, application of PBPK modeling used in conjunction with IVIV correlation approach can provide a useful tool to study pulmonary drug disposition and enhance understanding of drug transport in the lung.

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