

The Use of *In Vitro* 3D Cell Models in Drug Development for Respiratory Diseases

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1. Introduction

In a certain way, drug developers are like the blind men in the well-known tale of "THE BLIND MEN AND THE ELEPHANT", who believe the elephant to be like a water spout, a fan a pillar and a throne since they can only feel a different part but only one part of the elephant's body such as trunk, ear, leg, back. Impossible to test a drug lead on human beings, drug developers also are obliged to forge a whole picture of the "elephant" - how a drug candidate would behave in a whole human body, by using information from the "parts" - models. The task of the drug developers are far more complex and challenging than the blind men, instead of touching only the surface, they have to go deep into the human bodies: organs, tissues, cells, genes, proteins, lipids, hormones ... Furthermore, a living human being is a dynamic and interacting system, in a certain sense, the situation of the drug developers are even worse than the blind men: the blind men touch and feel a real elephant, the information that they get is true; a drug developer, most of the time, works on models, animal models or *in vitro* cell models, which are far from representing a human beings as a whole. As consequences, the information that one gets sometimes could be misleading. For drug developers, the misleading information could have serious consequences in terms of costs and human lives.

This blind men's approach may explain why a drug candidate, even though successfully passed pre-clinical stages, eventually failed at clinical trials. The failure of Torcetrapib, a drug developed by Pfizer, gives an example of just how difficult to develop a drug. Torcetrapib, designed to prevent heart attacks and strokes, is a cholesteryl-ester transfer protein (CETP) inhibitor. Genetic studies of the Japanese populations revealed that people with a deficiency of CETP presented a favorable lipid profile compared with unaffected family members: namely more High Density Lipoproteins (HDL, good) and less Low Density Lipoproteins (LDL, bad) (Inazu et al., 1990; Koizumi et al., 1991). CETP naturally became a target of drug development. Preclinical studies of Torcetrapib on different animal models (mice, rabbits, etc) didn't reveal any severe side effects (Tall et al., 2007). But, medication of Torcetrapib on human beings caused severe hypertension and an increased mortality; apparently there was no obvious beneficial effects on coronary heart disease. The development of Torcetrapib was halted at 2006.

This example illustrates another difficulty in drug development: the genetic heterogeneity of the human populations. The knowledge obtained from one group of people may not be applicable to another group of people. Quite often, this truth has been neglected.

Furthermore, human body is a complex dynamic system, with interconnected networks, equilibrium, feedback, compensation, redundancy, etc..., the cause-effect relationship between the target and the patho-physiological condition is not linear. In the example of CETP, people with the genetic deficiency of CETP, despite of having a favorable lipid profile, also suffer from coronary heart disease (Hirano et al., 1995).

Unfortunately, Torcetrapib is not an exception. According to a statistics, the failure rate at Phase III clinical trials is estimated to be 50% and only 12% of compounds entering into the human phase testing will eventually makes to the market place (Chuang & Stein, 2004).

However, despite of these difficulties, efficient and safe drugs have been successfully developed. The huge increase of the life-span of human populations is the testimony of this success.

The question now is how to improve the success rate of the drug development? In light of the above discussions, we could give a generally recommendations:

1. If possible, use models as close as possible to human patho-physiology and disease conditions.
2. Take into account of the genetic heterogeneity whenever possible during the preclinical studies.
3. Simulate as realistically as possible the dynamic and complex nature of biological responses.

The recent development of 3D human tissue models and the *in silico* models are some attempts to get closer to the human "reality". In this article, authors try to give an overview of the different models for studying the respiratory diseases and for drug development. First of all, in order to develop efficient and safe drugs, it is crucial to understand the nature and the underlying cellular and molecular mechanisms of the pathogenesis of the disease that one would like to treat. In the following sections, we will describe several major respiratory diseases.

2. Respiratory diseases

Respiratory disease is a medical term that encompasses pathological conditions affecting the normal function of the respiratory systems, making the gas exchange impossible. Anatomically, the respiratory system is composed of upper respiratory tract, trachea, bronchi, bronchioles, alveoli, pleura and pleural cavity, and the nerves and muscles of breathing. During the breathing, the respiratory system constantly expose to external insults such as bacteria, virus, particles, gas, etc... making the respiratory system highly vulnerable to various diseases. Indeed, according to the WHO World Health Report 2000, the top five respiratory diseases account for 17.4% of all deaths and 13.3% of all Disability-Adjusted Life Years (DALYs). Lower respiratory tract infections, chronic obstructive pulmonary disease (COPD), tuberculosis and lung cancer are each among the leading 10 causes of death worldwide. There are urgent and unmet needs of better treatments for respiratory diseases.

Respiratory diseases could be classified in various ways. In this article, we will classify them by the cause (etiology) of the disease. As such, the respiratory diseases can be divided into the following categories:

- Inflammatory lung disease
- Obstructive lung diseases
- Respiratory tract infections
- Respiratory tumors

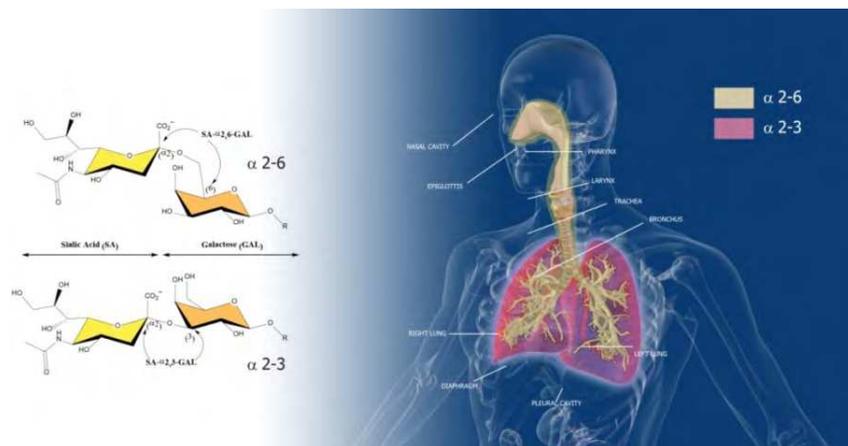


Fig. 2. Two types of linkages which plays an important role in viral tropism. Avian Influenza A viruses prefer $\alpha 2-3$ linkage, human Influenza A viruses, $\alpha 2-6$, porcine Influenza A viruses are adapted to both $\alpha 2-3$ and $\alpha 2-6$. In the human lung, there exists a particular distribution pattern of these two linkages: $\alpha 2-6$ linkage is present mainly on the airway epithelial surface lining the respiratory tracts, from the nose down to the bronchioles. In contrast, $\alpha 2-3$ linkage is located in the alveolar cavity mainly on the type-II cells.

The receptor of influenza A virus is glycosylated-proteins with a terminal sialic acid linked to galactose. There exist two main types of linkages: SA- $\alpha 2-3$ -Gal and SA- $\alpha 2-6$ -Gal. It has been shown that the avian Influenza A viruses prefer $\alpha 2-3$ linkage, human Influenza A viruses, $\alpha 2-6$, porcine Influenza A viruses are adapted to both $\alpha 2-3$ and $\alpha 2-6$.

What is interesting is the distribution of these two types of linkage in human airway epithelial cells. Using lectin probes specific for each type of linkages, the distribution of these two types of linkages has been studied in the human lungs (Shinya et al., 2006; Van Riel et al., 2006). It has been demonstrated that epithelial cells in the paranasal sinuses, the pharynx, the trachea, the bronchi as well as in the terminal and respiratory bronchioles, mainly express SA- $\alpha 2-6$ -Gal. In contrast, SA- $\alpha 2-3$ -Gal was found on non-ciliated cuboidal bronchiolar cells at the junction between the respiratory bronchiole and alveolus, and a substantial number of cells lining the alveolar wall also expressed this molecule. Moreover, the SA- $\alpha 2-3$ -Gal-positive alveolar cells also reacted to an antibody against surfactant protein A; this suggests that they were alveolar type-II cells (which express surfactant protein A) (Shinya et al., 2006; Van Riel et al., 2006).

Using similar approach, Varki and Varki confirmed these results. Moreover, they discovered that this distribution pattern seems to be unique of human lungs, it is even absent in the lung of great apes (Varki & Varki 2009).

Since most of the bacteria infect the human respiratory tracts without entering into the cells, the tropism of bacteria is less evident. But, in certain physiological as well as pathological conditions, bacteria preferentially infect certain species or tissues. As example, in patients suffering from cystic fibrosis (the pathology will be discussed below), the respiratory tracts of the patients are chronically colonized by *P. aeruginosa* which can rarely be eradicated. This chronic infection provokes lung inflammation and lung injury, leading to respiratory failure and death (Kerem et al., 1990). The reason of this persistent infection by *P. aeruginosa* is still

not clear. But it is reasonable to assume that the respiratory tracts of the CF patients provide a niche particularly favorable to *P. aeruginosa*.

So, given these particular and unique pathogen and host relationship, it is preferable to use human models for drug development for treating viral and bacterial infections of the human respiratory tracts.

2.2 Inflammatory lung diseases

Another category of respiratory diseases is the inflammatory lung diseases such as asthma, cystic fibrosis, emphysema, chronic obstructive pulmonary, characterized by a high neutrophil count. The mechanisms of immune responses in cystic fibrosis, asthma, COPD diseases have been extensively documented. The common feature of these inflammatory airway diseases is the involvement of the respiratory airway epithelia.

Cystic fibrosis (CF) is an autosomal recessive, a multisystem disorder, characterized primarily by defective electrolyte transport in **epithelial cells** and abnormally viscid mucus secretions from glands and mucus epithelia. The impairment of the mucociliary clearance leads to chronic infection and inflammation, ultimately causing cystic bronchiectasis, severe airflow obstruction and death (Boucher, 2004, 2007) (Figure 3).

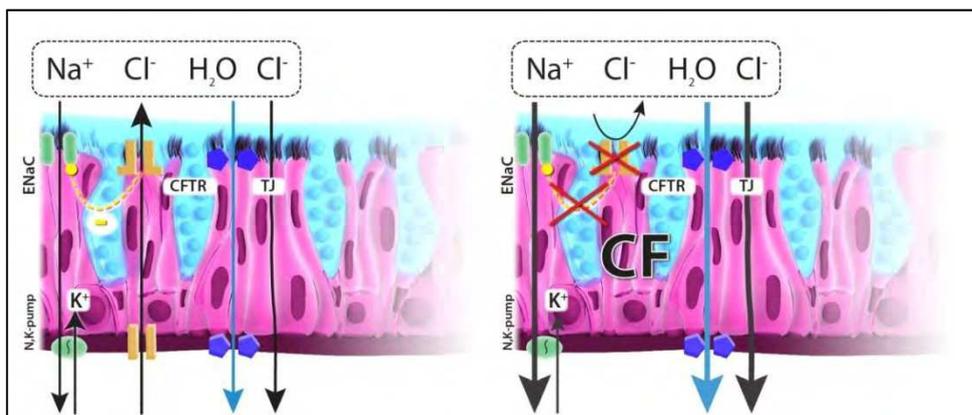


Fig. 3. Schema depicts normal airway epithelial ion transport, dysfunctional transport in CF. Normal airway epithelia (left): CFTR functions as a Cl^- and it also regulates Na^+ absorption (it inhibits ENaC). The quantity of NaCl on airway surface is optimally regulated to osmotically hydrate the periciliary and mucus layers allowing efficient mucus clearance. CF airway epithelia (right): In the absence of the CFTR protein in the apical membrane consequent to common CF mutations, such as ΔF508 CFTR, the CF epithelium can exhibit unrestrained Na^+ absorption and a failure to secrete Cl^- . These combined defects reduce the quantity of NaCl on CF airway surfaces, maintain less water osmotically on CF airway surfaces and, hence, lead to depletion of water in the periciliary liquid layer (PCL) and collapse of a thickened mucus layer onto the epithelial surface.

Asthma is an inflammatory disease characterized by allergic reaction to allergens and chemicals. The hallmark of asthma diseases is the antigen-specific IgE production. Even though the effector cells in asthma pathogenesis are immune cells like dendritic cells, helper

T cells (usually CD4⁺ cells), eosinophils, neutrophils, etc... the airway epithelia play an important role in the induction phase of asthma. Indeed, recent advances suggest that a cytokine named Thymic Stromal Lymphopoietin (TSLP) might play a key role in biasing the Th0 cells to Th2 differentiation pathway (Rochman et al., 2008; Ziegler et al., 2006; Esnault et al., 2008; Shi et al., 2008). What is more, TSLPR-knock-out mice failed to develop lung inflammation upon ovalbumin challenge (Al-Shami et al., 2005). Conversely, the over-expression of TSLP in mice induces spontaneous airway inflammation and atopic dermatitis (Zhou et al., 2005; Yoo et al., 2005), suggesting TSLP is an important factor necessary and sufficient for the initiation of allergic airway inflammation. The implication of TSLP in Th2 response is further confirmed by studies of other allergic diseases such as the skin atopic dermatitis (Zhou et al., 2005) and intestinal immune homeostasis (Zaph et al., 2007). Interestingly, expression of TSLP is elevated in the bronchial biopsies from the asthmatic patients compared to that of healthy donors (Ying et al., 2005).

Chronic Obstructive Pulmonary Disease (COPD) is now the fifth leading cause of death worldwide (Pauwels & Rabe, 2004) and it will become, as predicted by the World Health Organization (WHO), the third leading cause of death worldwide by 2030 (www.who.int/respiratory/copd/en/index.html). Even though airborne pollutants such as smoke from the burning fuel or coals can cause COPD, the main inducing factor is the cigarette smoke. COPD is a complex syndrome comprised of airway inflammation, mucociliary dysfunction and consequent airway structural destruction. This process is considered non-reversible.

Upon the irritant challenge, the airway epithelial cells synthesize and release pro-inflammatory cytokines and chemokines such as IL-8, MIP-3 α , which in turn recruit neutrophils, CD8⁺ T-lymphocytes, B-Cells, macrophages and dendritic cells to the lumen of the airways. The matrix-metalloproteinases (MMP-6, MMP-9), among other mediators, cause airway injury and remodeling, eventually leading to airway obstruction.

3. The Airway epithelium: Central to the pathogenesis of respiratory diseases

From the above descriptions, it is clear that the airway epithelial cells occupy a central position in the pathogenesis of most respiratory diseases, ranging from infectious diseases, genetic diseases, to most inflammatory diseases. Indeed, it has been long recognized that the airway epithelium is more than just a barrier: it synthesizes and releases a large panel of chemokines, cytokines, lipids, growth factors, proteases, protease inhibitors, for example, IL-8, IL-6, IL-17F, TGFs (Folkerts et al., 1998; Laberge et al., 2004; Suzuki et al., 2007). And the expression of these cytokines and chemokines are induced and modulated by various external insults like viral and bacterial infections, cigarette smokes (Nakamura et al., 2008) and are associated with disease conditions like CF, asthma, and COPD (Fig. 4).

This paradigm provides a theoretic framework for developing more relevant *in vitro* cells models of allergic asthma and COPD, especially the *in vitro* 3D cell models of the human airway epithelium.

4. The experimental models

A survey of the existing models of respiratory diseases may be useful for the readers. Knowing the strengths and limitations of each model will help drug developers to choose the more appropriate tools for their work.

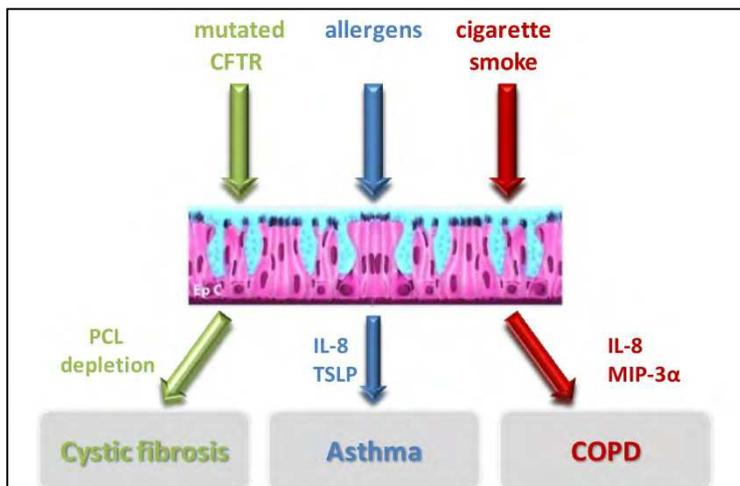


Fig. 4. The Airway epithelium - central to the pathogenesis of respiratory diseases. In addition to its barrier functions, the airway epithelium is also an immune-modulator involved in most of the respiratory diseases.

4.1 Animal models

Animal models are widely used for studying the respiratory diseases and for drug development. Due to the economical and technical reasons, the small rodents like mice and rats are the most popular and widely used animal models. Besides, chicken eggs, rabbits, guinea pig, cats, dogs, sheep, monkeys are also routinely used (Epstein, 2004).

For example, embryonated chicken eggs have been extensively used for growing the influenza viruses and for vaccine production (Potkin & Potkin, 2006; Artenstein, 2009). However, since the dominant linkage in the embryonated chicken eggs is α 2-3, some of the field strains grow poorly or not at all in chicken eggs. Furthermore, High mutation rates of RNA viruses like Influenza A allow the generation of viral escape mutants, rendering vaccines and drugs directed against virus encoded targets potentially ineffective (Neumann et al., 2009). A new strategy is to temporarily disable the host cellular factors necessary for viral replication, thus reduce the risk of selecting for resistant viral mutants. Using genome-wide RNAi screen approach, Karlas et al. have identified hundreds of human host factors crucial for influenza A virus (H5N1) replication in A459 cells (Karlas et al., 2010). However, Due to the genetic drift of A549, a cancerous alveolar cell line, it is necessary to confirm these results in primary human pneumocytes. As discussed above, the major concern about pandemic threat of avian influenza A is the possibility of avian flu virus to acquire the ability to infect the upper human airway tracts which are rich in α 2-6 linkage.

As animal models, mice and rats are routinely used for studying respiratory diseases. Besides the economical considerations, the biggest advantage of rodent models is the possibility to modify genetically the animals. Indeed, transgenic mice with knock-out CFTR or with delta-F508 mutations have been created (Colledge et al., 1992; Snouwaert et al., 1992; Ratcliff et al., 1993; O'Neal et al., 1993; Hasty et al., 1995). Unfortunately, these transgenic mice don't present lung problems as found in cystic fibrosis patients, highlighting once again the molecular, cellular and physiological differences between animals and human.

As for asthma and COPD animal models, since the rodents don't naturally develop these human diseases, allergic asthma or COPD has to be induced artificially by immunizing and challenging the respiratory tracts with antigen/allergen or proteases/cigarette smokes (for reviews: Epstein, 2008; Churg et al., 2008). Even though some asthmatic symptoms like eosinophil infiltration, mucus hypersecretion, airway hyper responsiveness, and elevated IgE production, can be observed, the lung inflammation is often transient rather than chronic as in human being. Similarly, due to the considerable differences of lung anatomy and susceptibility to injurious agents among species (Bracke et al., 2007), none of the animal COPD models reproduces the exact changes seen in humans (Churg et al., 2008).

Occasionally, naturally occurring animal disease models could also be found and used. For example, *Ascaris suum*-allergic sheep and flea-allergic dog have been discovered and used for studying asthma diseases and for drug development (Singh et al., 2002; Tang et al., 2003).

One way to get closer to human disease conditions, so-called humanized animal models have also been made to explore the pathogenesis of allergic asthma: the human peripheral blood mononuclear cells were isolated and injected into T and B lymphocyte deficient severe combined immunodeficiency (SCID) mice, creating a human-SCID mouse. Even though it is an innovative approach, due to the complexity of the human immune systems, it is not possible to humanize all the immune cells of the SCID mouse.

In short, animal models, even though useful, indispensable and informative, don't give the true picture of the human respiratory diseases. Sometimes, it is even misleading. Furthermore, none of these animal models can reflect the human genetic heterogeneity which is key for developing safe and efficient drugs.

4.2 Human respiratory disease models

There are several categories of human disease models:

- Cell lines
- Primary cells
- 3D cell models
- Cell co-cultures
- Explants
- *In silico* models

Until now, the most popular human models are cell lines derived from various human tissues.

4.2.1 Epithelial cell lines

The airway epithelia constitute the first line of defense against the external insults. It has a pseudo-layer structure consisted of three main types of cells: ciliated epithelial cells, mucus cells and basal cells. The mucus cells synthesize and secrete mucin-rich mucus which trap most of the inhaled particles, virus and bacteria, the later are eliminated from the body by muco-ciliary clearance via the cilia-beating. All the three cell types contribute to the pathogenesis of respiratory diseases, for example, inflammatory reaction, mucus hypersecretion, airway remodeling (Epstein, 2004; Verstraelen et al., 2008).

As airway epithelial cell lines, the most frequently used ones are A549, BEAS-2B, Calu3, 16HBE14o-, etc... The characteristics and uses of these cell lines have been nicely reviewed by Verstraelen et al., 2008.

A549

Origin: This line was initiated in 1972 by D.J. Giard, et al. (Giard, 1972) through explant culture of lung carcinomatous tissue from a 58-year-old Caucasian male. As type II pulmonary epithelial cells (alveolar pneumocyte), it synthesizes lecithin with a high percentage of disaturated fatty acids (surfactants). It is oncogenic when tested in nude mice.

Applications: The cells can be used to screen chemical and biological agents for ability to induce or affect differentiation and/or carcinogenesis. Mechanistic studies, pathway-mapping and target-finding, or ranking of the toxicity potency of chemicals.

BEAS-2B

Origin: BEAS-2B cells were isolated from normal human bronchial epithelium obtained from autopsy of non-cancerous individuals. The cells were infected and immortalized with an adenovirus 12-SV40 virus hybrid Ad12SV40 and cloned. These cells retain the ability to undergo squamous differentiation in response to serum, and stained positively for keratins and SV40 T antigen.

Applications: The cells can be used to test the toxicity of chemicals and biological agents relevant to upper airway epithelia. Suitable for mechanistic studies, pathway-mapping and target-finding.

Calu-3

Unlike most immortal cells, Calu-3 cells form sheets of cells that are welded to each other by tight junctions. These sheets form a fully functional epithelium that can transport large quantities of ions and fluid. In addition, Calu-3 cells have the highest level of natural CFTR expression of any known immortalized cell, even higher than some intestinal cell lines that once held the record. Thus, this cell line is suitable to study cystic fibrosis and drug discovery (Haws et al., 1994).

4.2.2 Immune cells (Effector cells)

Dendritic cells: Although both the skin and lung airway mucosa possess resident dendritic cells, the majority of studies conducted to date have utilized human peripheral blood mononuclear cell-derived dendritic cells (PBMC-DC) due to their relative ease of extraction and the ability to obtain larger quantities of cells (Casati et al., 2005). Several protocols have been established to generate human DC *in vitro*. Starting with blood or bone marrow-derived CD34+ hematopoietic progenitor cells (HPC), DC can be generated under various culture conditions with a cocktail of specific cytokines. Despite of the progress made in the field, it is still difficult to obtain sufficient amount of primary DC cell for basic or clinic research. Therefore, the use of cell lines such as THP-1, KG-1, especially the MUTZ-3, proves to be invaluable (Santegoets et al., 2008).

Mast cells, neutrophils, eosinophils, , basophils, are considered as effector cells which are involved in early and late phases of asthma by releasing a plethora of inflammatory mediators. Their roles in broncho-constriction, mucus secretion, and airway remodeling have clearly defined. Many therapeutics are targeting these effector cells and associated key molecules (Casale et al., 2008). These cells can be isolated from the blood or cord blood and cultured *in vitro*. Their behaviors such as migration, free radical production, viability and apoptosis, can be assessed after stimulation by allergen and cytokines (Frieri et al., 2003; Tang et al., 2003; Nilsson et al., 2004).

4.2.3 The drawbacks of cell lines

1. These cells have been transformed by oncogenes one way or other, thus certain signal transduction networks have been deregulated.
2. Genetic aberrations such as chromosomes loss, chromosomes translation, mutations, etc...
3. These cell lines cannot give rise to fully differentiated phenotypes of the original tissues such as cilia formation, mucus secretion, epithelium repair and remodeling.
4. Under the monolayer culture conditions, the cells behave totally different as they do in *in vivo* situations. These differences have been illustrated by comparing the responses of the same cancer cells to drugs (Bissell et al., 2006; Yamada et al., 2006; Griffith & Scharztz, 2006).

4.2.4 Fully differentiated 3D human airway epithelial models

To overcome these shortcomings of the cell lines, different techniques have been developed to make 3 dimensional (3D) cultures, by providing a micro-environment or architecture closer to *in vivo* situations. Among these techniques, cellular matrix scaffold, hang-drop culture, perfusion culture chambers, air-liquid interface cultures, etc...

However, in order to simulate the *in vivo* lung conditions, the ALI cultures seem to be more appropriate: the basal-lateral side of the epithelia is immersed in the culture medium and the apical side is exposed to humidified air with 5% CO₂. Furthermore, with the Costar PET Transwell inserts, it is very practical and convenient to use. It is suitable for most of the applications, such as imaging, immune-cytochemistry, toxicity tests, electrophysiological studies (Ussing chamber measurement), assessment of drug permeation and drug formulations, etc...

4.2.4.1 MucilAir

It is a fully differentiated and ready-to-use 3D model of human airway epithelium, constituted with primary human epithelial cells freshly isolated from the nasal or bronchial biopsies. It is commercially available and ready-to-use (Epithelix, www.epithelix.com). MucilAir (Fig. 5.), is not only morphologically and functionally differentiated, but also can also be maintained in a homeostatic state for a long period of time. Using cells from diseased donors, different versions of MucilAir can be made such as asthmatic, allergic, COPD, CF, etc...

4.2.4.2 Applications of MucilAir in drug development

Due to its fully differentiated nature, MucilAir can be used for studying various respiratory diseases. In the following paragraphs, we will give some examples about the applications of MucilAir in drug development.

4.2.4.2.1 Viral infections

MucilAir has been successfully used for studying Pandemic H1N1 2009 influenza virus (Brookes et al., 2010). It is also suitable for other respiratory viruses like Respiratory syncytial virus (RSV) and Human Rhinovirus (HRV) (Fig. 6.). The results are highly reproducible.

4.2.4.2.2 Electro-physiology of the airway epithelia (Ussing chamber measurements)

One of the characteristics of the airway epithelia is the active ion transport which is essential for regulating and maintaining the ion composition and height of the pericilliary liquid layer. The ion channel activities of the airway epithelia can be monitored in a modified Ussing Chamber. In the following figure, the short circuit current mediated by CFTR is

absent when stimulated with Isoproterenol which increases the intra-cellular cyclic AMP (see above discussion on CF pathology). Typical recording is shown in Fig. 7.. Related to electrophysiology is the muco-ciliary clearance. It is convenient to use MucilAir for evaluating the drug effects on cilia beating and muco-ciliary clearance.

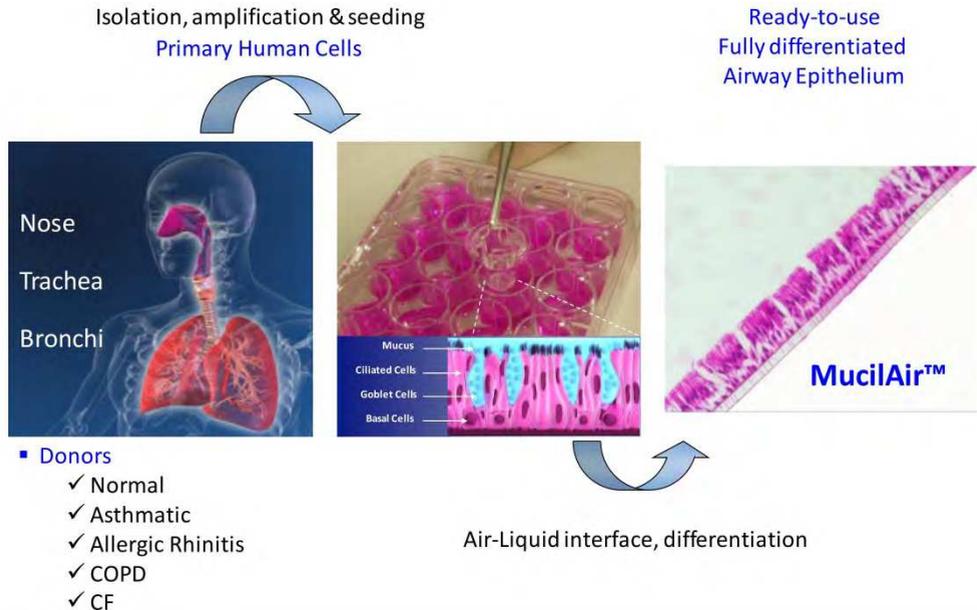


Fig. 5. MucilAir, a fully differentiated 3D *in vitro* cell model of the human airway epithelia. Epithelial cells were freshly isolated from the biopsies (nose, trachea, and bronchi), then seeded onto a semi-porous membrane (Costar Transwell, pore size 0.4 μm). After about 45 days of culture at air-liquid interface, the epithelia were fully differentiated, both morphologically and functionally. Depending on the pathology of the donors, different versions of MucilAir could be made.

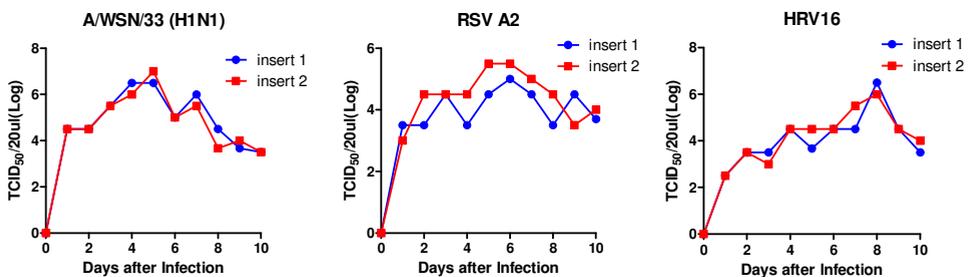


Fig. 6. MucilAir was infected apically: A/WSN/33, H1N1 influenza, 1MOI; RSV A2, 0.1MOI; HRV16, 1MOI. The virions were collected by washing the apical surface with 500 μl culture medium. The titre of the viruses was measured by ELISA.

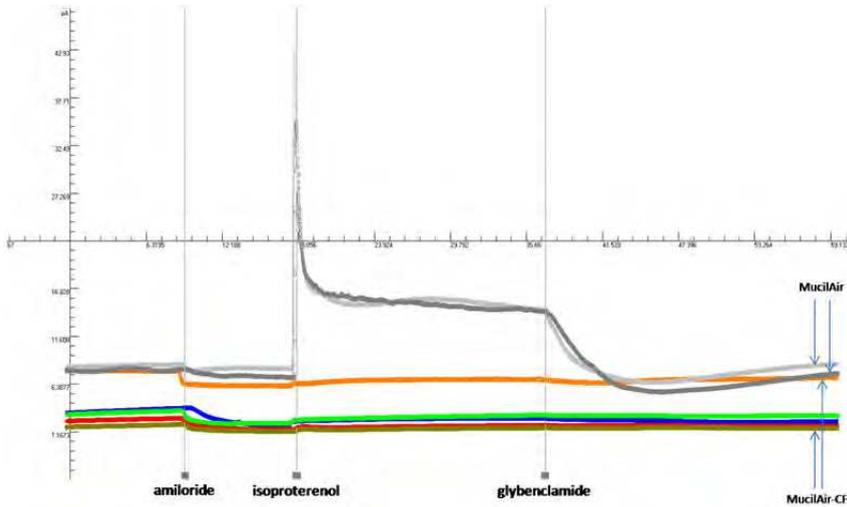


Fig. 7. Measurement of the short-circuit current in modified Ussing chambers. The reconstituted epithelia are placed in modified Ussing chambers and the short-circuit currents are measured after the addition of various blockers and activators of the ionic channels. Addition of Amiloride (blocker of the sodium channels) reduced the current. The addition of Isoproterenol increased the current in normal MucilAir, but in MucilAir-CF. The addition of glybenclamide (an inhibitor of CFTR channel) completely inhibited the currents mediated by CFTR in normal MucilAir.

4.2.4.2.3 Mucociliary clearance analysis

The muco-ciliary clearance is the principal defense mechanism of the respiratory systems. The impairment of this function is the major cause of chronic infection and inflammation, as exemplified in cystic fibrosis disease. For experimental reasons, direct measurements of mucus velocity in human lungs are presently available only for the trachea, with values ranged from 4-6 mm/min. (Hofmann et al., 2002). The only reported value of the mucus velocity in the main bronchi is about 2.4 mm/min (Foster et al., 1982). Interestingly, the same value has been obtained by using an *in vitro* culture model of the bronchial airway epithelial cells (Masui et al., 1998). However, the velocity of muco-ciliary clearance varies considerable depending on individuals, anatomical location, and disease conditions, etc... Indeed, the values that we obtained from the nasal epithelia are slightly inferior to that of bronchial ones, about 1.8 mm/min (Fig. 8). Due to the simplicity of *in vitro* methods, it is possible to access the drug effects on muco-ciliary clearance in various disease settings.

4.2.4.2.4 Multi-endpoint test strategy

In addition to its applications in viral infection and cystic fibrosis, MucilAir can also be used for testing the toxicity and efficacy of the drugs candidates. To this end, a multi-endpoint strategy is used (Fig. 9). The drug candidates can be applied on the apical surface as liquids, solids, gaz, smoke and nanoparticles. The effects of drugs can be monitored by several endpoints: such as the trans-epithelial electric resistance (TEER), cell viability tests (Resazurin, LDH), cilia beating frequency monitoring, muco-ciliary clearance, mucus secretion, release of cytokines/chemokines.

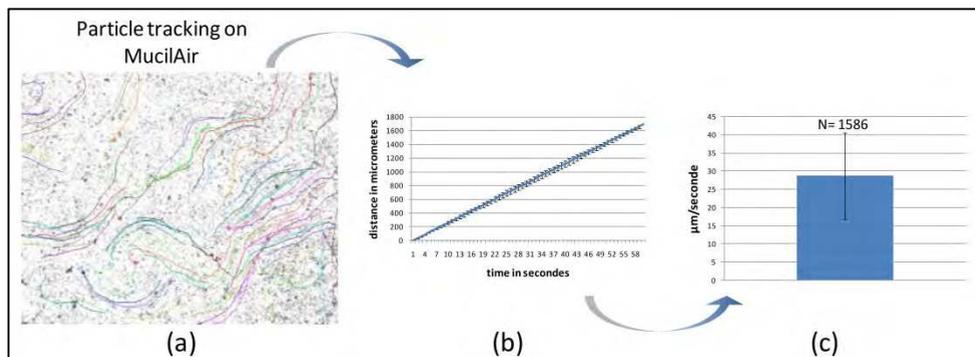


Fig. 8. Due to its fully differentiated nature, mucociliary clearance can be easily studied *in vitro* on MucilAir. Briefly, the mucociliary clearance is monitored using a fully automated setup dedicated to video-microscopy. Microbeads of 5 microns are seeded onto the apical surface of MucilAir-normal or MucilAir-CF cultures. Then, pictures are taken every second during 1 minute in order to reconstitute movies showing the movement of the small particles. The particle movement is then tracked using dedicated software for calculating velocities of beads' movement.

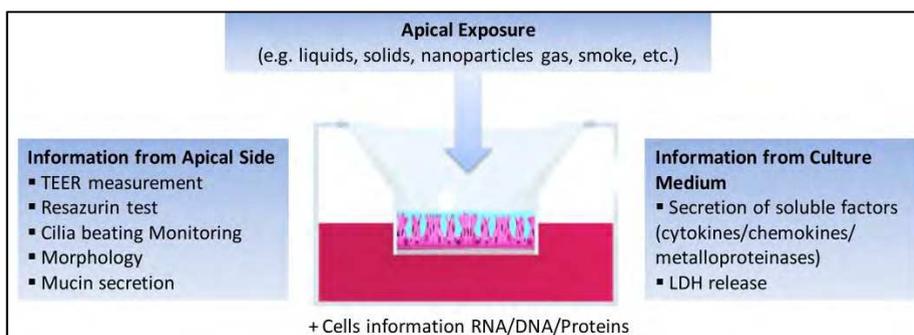


Fig. 9. Multiple end points testing strategy for toxicity testing using MucilAir

4.2.4.2.5 Dextran application

One of the problems that drug developers frequently encounter is the solubility of the compounds: not all the chemicals are soluble or stable in solutions. It is also difficult to test powders or nanoparticles. In order to solve this problem, a procedure which allows testing all kinds of solid substances was developed (PCT/IB2010/053956). The idea is to use inert and neutral substances as carrier. Dextran was successfully used as carrier to deliver the insoluble chemical compounds and nanoparticles onto the apical surfaces of MucilAir. Dextran is a bacterial byproduct; the dextran macromolecule consists of glycan groups linked end to end. It didn't show any harmful effect on MucilAir. The following figure describes the procedure and application of the dextran tablets (Fig. 10).

4.2.4.2.6 Simulation of chronic airway inflammation

Airway epithelium is more than just a barrier; it is also an immune-modulator. Upon external stimulations, it synthesizes and releases a large panel of chemokines, cytokines,

lipids, growth factors, proteases, protease inhibitors. In the following figure (Fig. 11.), the epithelia were repetitively stimulated by Cytomix (TNF- α /LPS). It is remarkable that the airway epithelial cells could recover after repetitive challenge of Cytomix, a relatively physiological stimulus. It was not the cases with some chemical compounds (data not shown).

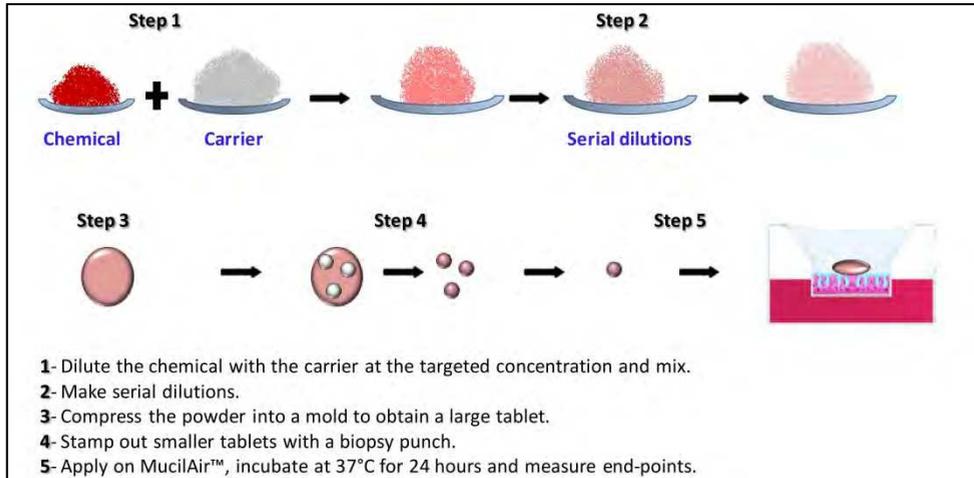


Fig. 10. Preparation and application of the Dextran Tablets

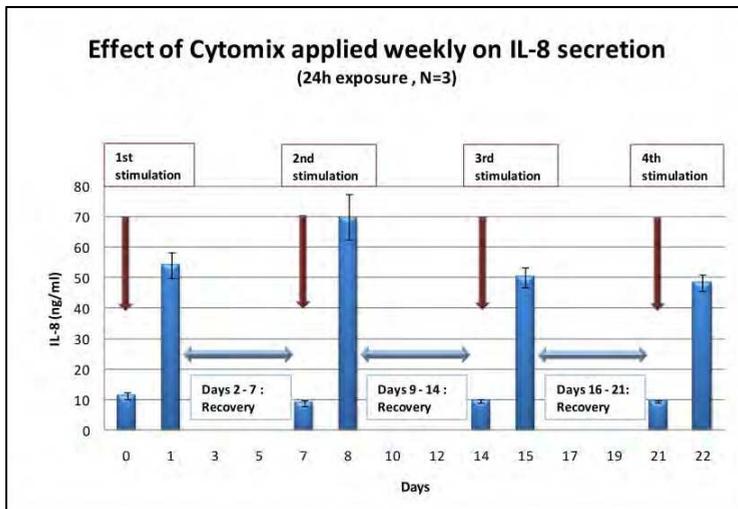


Fig. 11. Inflammatory response of the epithelia. Before the stimulation, the basal level of IL-8 was about 10 ng/ml. After 24 hours of stimulation with pro-inflammatory mediators (Cytomix = TNF- α + LPS), the amount of IL-8 released was increased five-fold. Upon removal of the stimulus, the amount of IL-8 returned to basal level as day 0. The epithelia could respond to the stimulus in a physiological manner again and again.

4.2.4.2.7 Repeated dose and long term toxicity testing using MucilAir

Quite often, it is necessary to perform repeated dose and Long term toxicity/efficacy test of drug candidates. Up to now, this kind of experiments can be performed only on animals. In the following graph, we provide a proof-of-concept for repeated dose tests using an *in vitro* cell model. It is a transposition of the OECD412 guideline for 28 day test on rodents. The TEER was used as endpoint, which is an indicator of the epithelial integrity (Fig. 12.). This is a very sensitive endpoint. The “No Observed Adverse Effect Level”(NOAEL) can be determined (around 10 mM).

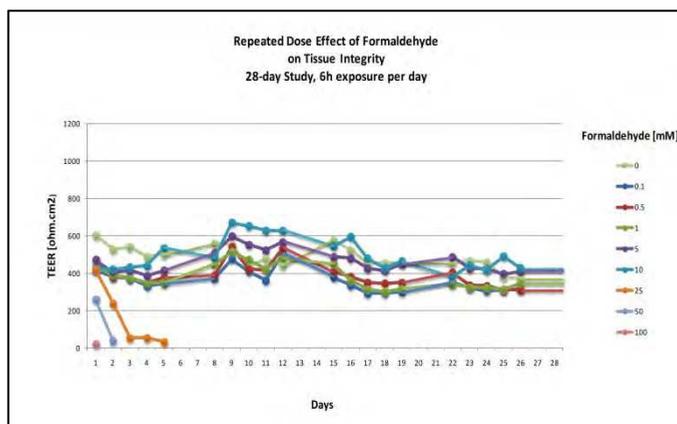


Fig. 12. Repeated dose tests of formaldehyde on MucilAir (28 days repeated dose exposure study). 6 hours per day exposure on MucilAir to Formaldehyde for a period of 28 days. Every day, tissue Integrity (TEER) were measured (N=3) then epithelia were reused for the next exposure.

4.2.4.2.8 Drug permeation

In recent decades, the nasal mucosa has become an established administration site for systemic as well as local drug delivery (Ugwoke et al., 2005). For developing novel drugs intended for this route, reliable methods are needed for assessing the rate and extent of absorption across the nasal epithelium. Desired methods should enable studies of enhancer effects and toxicology. By transposing the Caco-2 protocol (Hubatsch et al., 2007) to MucilAir™, trans-epithelial permeability of drug candidates or xenobiotics can be assessed on MucilAir™. Since epithelium is grown on separable inserts, kinetic studies are facilitated (Fig. 13.). The results obtained are very similar using different batches of MucilAir™ derived from different donors. Apical to basolateral and basolateral to apical permeability studies on height reference compound were performed (Table 1).

4.2.4.2.9 The problem of variability

One of the concerns of using the primary human cells is the variability from donor to donor: researchers don't like variability! Indeed, one of the gold standards of scientific analysis is the reproducibility. If one performs an experiment with a standard operating protocol, one should get the same results. We agree with this principle. But, the genetic heterogeneity of the human population is a reality that drug developers have to face sooner or later: Exposed to the same allergens, only a small percent of people develop asthma. Not all the smokers

suffer from COPD. This reality has to be taken into account whenever possible, and as early as possible. Thus, in our opinion, the donor-dependent variation is not a problem; on the contrary, it could be a solution for reducing failure rate at later stages. Using a large collection of frozen primary airway epithelial cells, it is possible to address the issue of the genetic heterogeneity of the human populations. With tools like genomics, proteomics, bioinformatics, etc, it would be possible to pin down cellular and molecular mechanisms underlying the donor to donor variability.

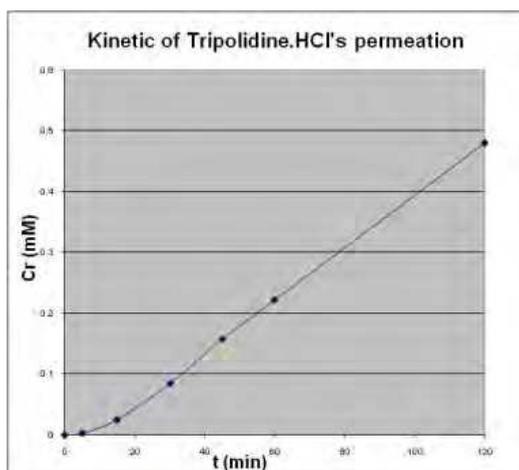


Fig. 13. Time course of the rate of permeability of Tripolidine.HCl from the apical to basolateral side (N=3).

Molecules	Papp (cm/s) A→B	Papp (cm/s) B→A	Asymmetry Ratio
Salicylic Acid	7.7×10^{-5}	1.7×10^{-5}	0.2
Nicotine	2.1×10^{-5}	3.3×10^{-5}	1.6
Propranolol. HCl	1.2×10^{-5}	1.6×10^{-5}	1.3
Ibuprofen	1.1×10^{-5}	1.9×10^{-5}	1.7
Tripolidine. HCl	9.7×10^{-6}	1.2×10^{-5}	1.2
Tetracaïne. HCl	8.0×10^{-6}	1.1×10^{-5}	1.3
Dopamine. HCl	3.0×10^{-6}	2.5×10^{-6}	0.8
Atenolol	2.2×10^{-6}	6.7×10^{-6}	3.0

Table 1. Evaluation of transport of 8 reference compounds across MucilAir (N=3)

4.2.5 Immune competent 3D models

Despite the central role of the airway epithelia, other cellular components are also implicated and necessary for understanding the pathology of respiratory diseases, especially the immune cells.

Co-culture systems: To study the cross-talks between different types of cells, the simplest way is by co-culturing different cells involved *in vitro*. Such experiments have been carried out using established cell lines: for examples, BEAS-2B are co-cultured with human lung fibroblasts (HFL-1 or W1STAR-38) (Lang et al., 1998), human umbilical vein endothelial cells (ECV304) (Mögel et al., 1998), or eosinophils (Wong et al., 2005), and primary human BECs with alveolar macrophages (Ishii et al., 2005). Using 3D cell models like MucilAir™ and freshly isolated immune cells, more reliable and faithful co-culture systems could be developed for studying the inflammatory respiratory diseases like asthma or COPD diseases and for drug development.

Explants: Another way is to use explants. In other words, part of animal or human lung was excised and cultured *in vitro* for a period of days or weeks. The effects of cytokines or chemical compounds on broncho-constriction have been studied (Wohlsen et al., 2003). Using explants models it is possible to assess immunomodulatory effects (Tan et al., 2009) and understand signaling processes involved in tissue hyperresponsiveness related to asthma (Morin et al., 2005). Precision Cut Lung Slides (PCLS) are now used for studying the effects of sensitizers. PCLS mirrors the complex interplay of different cell types in the living organ and allows physiological processes to be mimicked (Henjakovic et al., 2008). The problem with the lung explants is the rapid degradation of the tissue slices. It is also difficult to obtain health, normal tissues.

Lung on a chip: Ideal is to reconstitute the lung on a chip. Some attempts have been made. For example, Ingber's lab designed and constructed a microfluidics system, so-called Lung on a chip that reproduces both the lung's alveolar-capillary interface and the mechanical effects of breathing on that interface—all on a polymer chip about 2 cm long (Huh et al., 2010). Another example is a so-called **Multi-Compartmental Bioreactor** designed on the basis of allometric scaling laws in order to recreate physiological life conditions of four different human cell types (pancreatic, adipocyte, endothelial and hepatic cells), interconnected each other through media flow (Vozzi et al., 2011). Some physiologically relevant results have been obtained by Vozzi and coworkers, suggesting that interactions, mediated by metabolites present in media flow, have a remarkable effect more than the physical interaction and can lead to a restoration of physiological cell life conditions.

By incorporating the immune cells into these microfluidics systems, it is possible to build an immune-competent human *in vitro* lung model for studying the inflammatory respiratory diseases like asthma and COPD.

4.3 *In silico* models: A systems biological approach - virtual cells, virtual organs, and virtual patients

Despite the spectacular successes of molecular biology, some scientists did feel the drawbacks of the "blind men's approach", and they tried to take a more holistic viewpoint to describe the biological systems (von Bertalanffy, 1968; Noble, 2006). Back to 1960, Noble already developed the first computer model of the heart pacemaker (Noble, 1960). From 2000 onward, so-called systems biology has been gaining popularity and importance. The systems biology tries to put the parts - head, ear, trunk, and legs - together to form a whole

elephant: namely to achieve a holistic, quantitative, and predictive understanding through mathematical models that enable an iterative cycle between prediction and experiments (Sauer et al., 2007). Based on systemwide component identification and quantification ("omics" data) at the level of mRNA, proteins, and small molecular weight metabolites, Ishii et al. made a "virtual cell" which includes both the constituting components and the functional state of a metabolic network, providing a proof-of-concept for integrating a heterogeneous data set into a coherent whole (Ishii et al., 2007).

With increasing calculation capacity and better software, it is now possible to simulate more realistically the biological processes at all levels: molecular interaction (ligand-receptor), virtual cells, virtual organs, and even virtual patients (Epstein et al., 2004). The *in silico* approach allows to rapidly integrating new data and novel knowledge, and it can get better and better with time. It is reasonable to hope that in the near future, based on systemwide data collection using relevant human 3D cell/tissue models, we could build a virtual lung for studying the respiratory diseases and for new drug development.

5. Conclusion

Each model has its strengths and weaknesses and there is no perfect respiratory disease model. Depending on the goal or on application, one model could be better than another. Since the drugs are to be used by human beings, it is logic to assess the toxicity and efficacy on cells of human origin, especially on 3D human models. Due to its central role in pathogenesis of respiratory diseases, the 3D *in vitro* cell models of the human airway epithelium deserve more attention in the future. We believe that the 3D cell models like MucilAir are highly relevant and valuable for development of new drugs.

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7. References

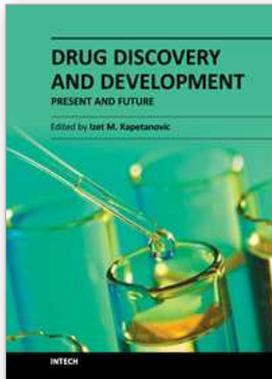
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Drug discovery and development process aims to make available medications that are safe and effective in improving the length and quality of life and relieving pain and suffering. However, the process is very complex, time consuming, resource intensive, requiring multi-disciplinary expertise and innovative approaches. There is a growing urgency to identify and develop more effective, efficient, and expedient ways to bring safe and effective products to the market. The drug discovery and development process relies on the utilization of relevant and robust tools, methods, models, and validated biomarkers that are predictive of clinical effects in terms of diagnosis, prevention, therapy, and prognosis. There is a growing emphasis on translational research, a bidirectional bench to the bedside approach, in an effort to improve the process efficiency and the need for further innovations. The authors in the book discuss the current and evolving state of drug discovery and development.

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