Expert Opinion

Preclinical models for pulmonary drug delivery

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Background: The lung comprises an interesting route of administration not only for topical drugs but also for systemically acting drugs. Over the last years, several models have been developed in order to study the efficacy and safety of pulmonary drug delivery. *Objective*: This review describes relevant drug delivery models for preclinical evaluation of inhaled drug products. *Methods*: Epithelial cell culture models, the isolated perfused lung and *in vivo* models are reviewed. The suitability and limitations of each method are discussed. This review is mostly based on publications from the last 10 years. *Results/conclusion*: Cell cultures are ideal models to compare transport rates of molecules and to study their mechanisms of transepithelial transport. Yet the most complete assessment of pulmonary drug delivery including delivery efficacy and safety remains provided by studies performed in vivo in animal models.

Keywords: cell cultures, inhalation, intratracheal instillation, isolated perfused lung, large mammals, pulmonary deposition, small rodents

Expert Opin. Drug Deliv. (2009) 6(11):1231-1245

1. Introduction

1.1 Drugs for local administration

The pulmonary route has been used for local administration of drugs for many years to treat lung diseases such as asthma and chronic obstructive pulmonary disease (COPD) [1]. Pulmonary drug delivery allows local drug targeting, and thereby administration of low doses and decreased drug concentrations systemically, resulting in reduced systemic side effects. In addition to β_2-agonists, corticosteroids, antibiotics and mucolytics, the licensed topical drugs, new classes of drugs are being studied for direct administration to the lungs. Systemic chemotherapy in primary or metastatic lung cancer shows low clinical efficacy, which might be related to low drug penetration locally in the tumour. Aerosolised chemotherapy could increase exposure of the lung tumour to the chemotherapeutic agent, while minimising systemic side effects [2]. Another example of local drug administration is pulmonary gene therapy where DNA or RNA interference is delivered. Potential applications include treatment of gene disorders such as cystic fibrosis, inflammatory diseases such as asthma and COPD, infections and cancer [3,4]. Administration of vaccines to the lungs is an efficacious strategy to induce mucosal as well as systemic immunity against infectious agents that are inhaled and cause, for example, tuberculosis, measles or flu. The pulmonary route is the only non-invasive route to provide systemic immune responses to a vaccine equivalent to injection at identical doses. Pulmonary vaccination might be especially interesting for mass-immunisation campaigns [3,5-7].

1.2 Drugs for systemic administration

In the last two decades, the lung has also been investigated as a 'needle-free' route for systemic administration of drugs. The lung has special anatomical and physiological features that are very interesting for drug delivery to the bloodstream, including the large epithelial surface area, the thin alveolar epithelium and the high vascularisation (**Table 1**) [8].

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Although the alveolar epithelium is tighter than the intestinal epithelium [9], the local enzymatic activity is lower, and there is no first-pass hepatic metabolism following pulmonary drug delivery. Both the lung and the intestine present an important epithelial surface area available for absorption but the drug deposits on the entire alveolar surface all at once, while it successively reaches intestinal segments [8,10].

Pulmonary drug delivery offers a non-invasive alternative to drug injection or oral drug administration. Small molecules can be absorbed rapidly from the lung with elevated bioavailability. Fast drug delivery is particularly beneficial to relieve acute symptoms such as pain, migraine and nausea. Examples of small molecule drugs include the opioids (morphine and fentanyl) for treatment of pain, or ergotamine for the treatment of migraine [8,11,12]. Therapeutic peptides and proteins are better absorbed from the lung than from any other non-invasive route of drug administration. In January 2006, the Food and Drug Administration (FDA) and European Medicines Agency approved an inhaled insulin product (Exubera), a fast-acting insulin for treatment of type 1 and type 2 diabetes. Yet, < 2 years after introducing the drug on the market, Exubera was withdrawn because of disappointing sales. Inhaled insulin provides the same control of glycaemia as insulin administered subcutaneously [8]. Yet, Exubera is more expensive. The dry powder inhaler device used to deliver insulin had the size of a tennis-ball-can and was therefore cumbersome. Lately, inhaled insulin has been related with an increased incidence of lung cancer cases among former smokers [13]. Pulmonary delivery of systemically acting drugs would therefore benefit from careful selection of drugs (with no growth factor properties as insulin has) as well as from further optimisation of inhaler size and costs in order to obtain more convenient and less expensive treatments. In the literature, research has been done on pulmonary administration of growth hormone [14], parathyroid hormone [15], erythropoietin [16] as well as other proteins [8].

1.3 Barriers to pulmonary drug delivery

The lung is in daily contact with significant amounts of pathogens and particles that can be harmful to the tissue. It has been estimated that the human airways are exposed to > 7 kg of pollutant a year [17]. The lung possesses different barriers to avoid particles carried within the airstream invading the lung. These barriers also decrease the amount of drug that reaches the lung and that is available for a local effect or for systemic absorption. The first obstacle to particle penetration in the lung is the airway geometry where successive bifurcations encourage particle impaction [18]. The relative humidity within the airspaces approaches 90% during the inspiratory phase and causes hygroscopic particles to grow in size and deposit [18,19]. Once deposited in the airways, insoluble aerosol particles and solubilised compounds can be removed by mucociliary clearance. The mucus is a viscoelastic gel composed of 95% water, 2% mucin, 1% salts, 1% albumin, immunoglobulins and enzymes and < 1% lipids [20]. Mucin is a high-molecular-mass glycoprotein with oligosaccharide side chains attached to a protein backbone and its fibre structure provides the gel consistency of mucus. The mucus forms a 5 – 55-μm-thick bilayer on the airway surface, with a periciliary fluid layer responsible for lubrification of cilia beating and an upper gel layer trapping particles [21,22]. Mucociliary clearance involves the movement of mucus (and entrapped material) resulting from ciliary beating from the airways to the oropharynx, where it is swallowed or expectorated [23].

In the alveolar region, the epithelium is protected by a 20 – 80-nm-thick surfactant lining fluid [24]. Pulmonary surfactant is composed of 80% phospholipids, 5 – 10% neutral lipids and 8 – 10% proteins. At the air–liquid interface, phospholipids form orientated monolayers that reduce surface tension. The pulmonary surfactant also has an important role in innate immune defence [25,26]. Delivered proteins have been suggested in some cases to interact with lung surfactant and aggregate [27,28]. Small insoluble particles that deposit in the alveoli are rapidly taken up by alveolar macrophages by means of phagocytosis or 'cell eating' [29]. Alveolar macrophages are a barrier to the transport of large proteins from the airway lumen into the bloodstream as well [30,31]. Large proteins (≥ 40 kDa) are slowly transported across the alveolo-capillary barrier and can remain within the airspaces for several hours. This gives time for alveolar macrophages to engulf them by pinocytosis or 'cell drinking', the uptake of soluble compounds and fluids [30]. By contrast, alveolar macrophages have no impact on pulmonary absorption of small proteins and peptides (≤ 25 kDa), which are cleared from the airspaces within minutes [31].

Lung epithelia are barriers to drug transport to underlying tissues (e.g., the airway smooth muscles targeted by β_2-agonists) as well as to the bloodstream. Cells from the airway epithelium are very different from those of the alveolar epithelium, as is the thickness of the epithelium in each region (**Figure 1**). The airway pseudostratified epithelium is made of several cell types and principally of the ciliated columnar cell, the goblet or mucus-secreting cell, the basal or progenitor cell and the Clara cell. The Clara cell is found in bronchioles, secretes glycoproteins and is a progenitor for ciliated cells. The airway epithelium is ~ 80 μm thick at the trachea but thins down to 10 μm at the bronchioles (**Figure 1**) [19,24,32]. The alveolar epithelium comprises type I and type II pneumocytes. Owing to their large apical surface and thinness (0.05 μm), type I alveolar cells cover > 90% of the alveolar surface [24,33]. The small compact type II cell produces the lung surfactant and is a progenitor for the type I cell [19]. Epithelial cells in the lung are intimately connected by several proteins forming tight junctions and presenting a paracellular barrier to drug absorption [9,24,34]. Compounds essentially hydrophilic and ≤ 40 kDa are principally transported across biological membranes by means of diffusion-limited paracellular pathways. Pinocytosis and receptor-mediated transcytosis can become significant for macromolecules > 40 kDa [24,35].

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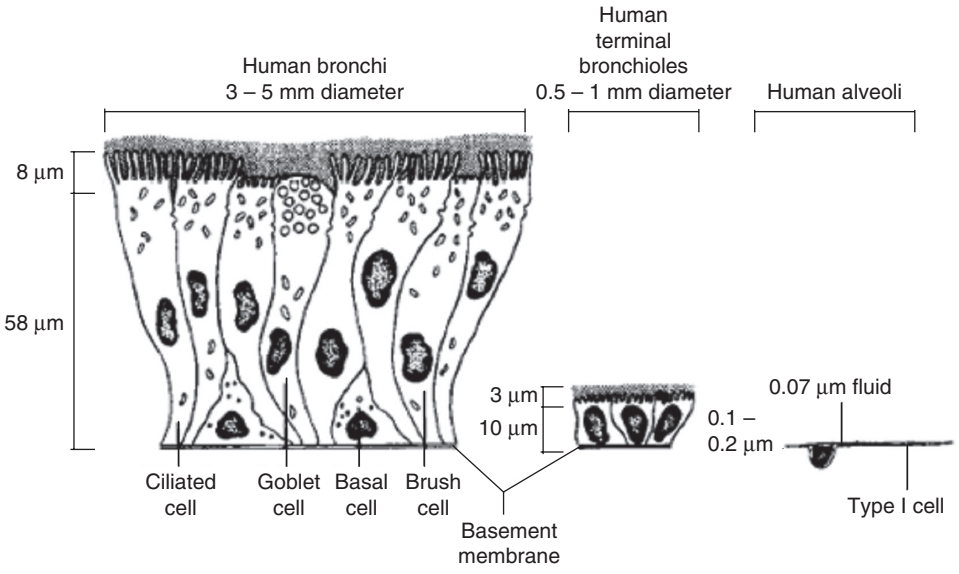

Figure 1. Schematic illustration of a lateral view of epithelial cells in the different regions of the human lung with the relative cell size and the surface fluid thickness.
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Table 1. Anatomical characteristics of mammalian lungs [96,125].

Characteristics	Human (70 kg)	Dog (10 – 15 kg)	Rabbit (2.5 – 3.5 kg)	Guinea-pig (0.4 kg)	Rat (0.25 – 0.35 kg)	Mouse (0.02 – 0.04 kg)
Turbinate complexity	Simple	Very complex	Complex scroll	Complex scroll	Complex scroll	Complex scroll
Lung weight (g)	1000	100	18		1.5	0.12
Lung symmetry	Dichotomous	Monopodial	Monopodial	Monopodial	Monopodial	Monopodial
Lung volume (ml)	4341	736	79.2	13	8.6	0.74
Surface area of the alveolar region (m^2)	143	40.7	5.8		0.4	0.07
Diameter of alveoli (µm)	219	126	88	65	70	47
Alveoli number ($\times 10^6$)	950	1040	135	69	43	18
Alveolar macrophages ($\times 10^6$)	5990	3940	142	58.8	29.1	2.9
Lining fluid volume (ml)	20 – 40 [126]	16.7 [127]	1.22 [128]		0.045 – 0.055 [110]	0.005 – 0.015 [129]

2. Models for pulmonary drug delivery

2.1 Cascade impactors

Cascade impactors measure the aerodynamic behaviour of aerosol particles by size-separating the dose in impactor plates. Cascade impactors yield useful aerosol parameters such as the mass median aerodynamic diameter (MMAD) and the fine particle fraction (FPF). The FPF is the percentage of the drug mass contained in ≤ 5 µm MMAD particles [36]. *In vitro* particle sizing data obtained from impactors aim first at controlling the quality of the pharmaceutical product and second at providing an analysis tool for product development. It is the method of reference of the FDA for stability testing of inhaled drug products.

It is expected that results from cascade impactors predict human lung deposition data as particle aerodynamic size determines aerosol deposition in the human respiratory tract [37]. Although impactor data correlate well with lung deposition data in humans obtained by gamma scintigraphy, in general the FPF systematically overestimates whole lung deposition in humans [38]. The cascade impactor does not represent the respiratory anatomy and, in particular, the inlet throat to an impactor does not adequately mimic the anatomical complexity of the human upper airway. Moreover, the aerosol

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enters the cascade impactor at a constant airflow rate, which does not take into account the variations in human respiratory airflow that occur during inhalation. Measurements in cascade impactors are made at room temperature and at low relative humidity, which is not representative of human airways' ambient conditions [37].

2.2 *In vitro*

The *Principles of Human Experimental Technique*, published by Russels and Burch, implemented the 3 Rs rule, 'replace, reduce and refine', which are widely spread as guidelines for animal experimentation [39]. In this respect, *in vitro* models for pulmonary drug delivery studies offer a very interesting alternative as they bring up fewer ethical questions but also because they allow a rapid screening of drugs.

Several cell culture models of the respiratory tract are described in the literature using both continuous and primary cell cultures. In both cellular models, it is important that epithelial cells form a tight monolayer in order to represent the natural epithelial barrier. Monolayer tightness and integrity are classically assessed by measuring transepithelial electrical resistance (TEER) and potential difference across the monolayer. Monolayers of lung epithelial cells allow the characterisation of drug transport and assessment of potential drug and formulation toxicity. Drug transport is classically measured in the apical to baso-lateral direction, and vice versa, in order to check for active transport mechanisms. The apparent permeability coefficient (P_{app} in centimetres per second) is a key characteristic of drug transport and is defined as:

$$P_{app} = \frac{J}{C_0}$$

where J (in micrograms per square centimetre per second) is the drug steady-state flux across the monolayer and C_0 (in micrograms per cubic centimetre) the initial donor concentration of the drug. Toxicity can be assessed by measuring TEER and by using the MTT assay [40-44].

2.2.1 *Continuous cell cultures*

Continuous cell cultures are more reproducible and easier to use than primary cell cultures but they often do not have the differentiated morphology and the biochemical characteristics of the original tissue [45].

There are few cell lines derived from alveolar epithelial cells. A549 is a type II alveolar epithelial cell line that originates from human lung adenocarcinoma. It can be very useful in metabolic and toxicological studies but it is less interesting as a drug delivery model because A549 cells do not form tight monolayers. Indeed, TEER values are < 200 Ω cm^2 (**Table 2**) [46,47].

Cell lines derived from bronchial epithelial cells are often used as *in vitro* models of pulmonary drug delivery. The Calu-3 cell line derives from bronchial epithelial cells of a human adenocarcinoma. In contrast to A549 cells, Calu-3 cells form tight monolayers with TEER > 1000 Ω cm^2, and immunocytochemical staining shows the expression of tight junctional proteins such as zonula occludens protein-1, occludin and E-cadherin (**Table 2**) [48]. Low activity levels of P-glycoprotein, the prominent drug efflux transporter, were detected in Calu-3 cells [49]. The cell line 16HBE14o- has been generated by virus transformation of normal bronchial epithelial cells. 16HBE14o- monolayers show good resistance with high TEER values (up to ~ 800 Ω cm^2). The presence of tight junctions was also confirmed by immunofluorescence staining of tight-junctional proteins [50].

2.2.2 *Primary cell cultures*

As cultures of primary cells present cell characteristics and state of differentiation more similar to the *in vivo* situation, they are extremely useful for drug transport studies. Yet they are costly models, time-consuming owing to cells' isolation from the lung and the monolayer presents a lifetime of only a few days. Moreover, tight monolayers with high TEER are not easily obtained and involve significant know-how.

Most primary cell cultures used as models for pulmonary drug delivery and transport studies consist of alveolar epithelial cells. Type II pneumocytes for primary culture can be isolated from the lung of different species. Human cells are the most representative of the clinical situation, but they are less accessible than cells from other mammals. Human type II pneumocytes are isolated from normal lung tissue of patients undergoing partial lung resection. In culture, the cells undergo differentiation into type I-like cells, as indicated by morphological and histochemical changes [34,51]. In early stages of the cell culture, the cells produce high levels of surfactant protein C and low levels of caveolin 1, a marker of type I pneumocytes, and conversely at later stages. On day 8 of culture, the cells form a tight monolayer consisting mainly of type I cells and some interspersed type II cells, with TEER > 2000 Ω cm^2 and potential difference > 10 mV (**Table 2**) [51].

The most utilised model has been rat alveolar epithelial cell monolayers because rat tissue is easily accessible. It has permitted assessment of the transport rates of small molecules [52], dextrans [35], peptides and proteins [53,54]. It has also been used to study mechanisms of transepithelial transport [55] and to test strategies to increase transport. These cells form tight monolayers as human alveolar epithelial cells do, with TEER > 2000 Ω cm^2 and a potential difference > 10 mV (**Table 2**). Dodoo *et al.* used monolayers of rat alveolar cells of varying electrical resistance to measure the apparent permeability coefficient (P_{app}) of mannitol and showed that decreasing TEER from 2450 to 1200 Ω cm^2 did not greatly affect mannitol permeability, whereas decreasing it further affected it greatly (**Figure 2**) [56]. Therefore, monolayers of type I-like cells with a lower TEER limit of 1200 Ω cm^2 can be proposed for transport studies.

Recently, porcine alveolar epithelial cells in primary culture were characterised and used as a model for drug transport studies. Compared with human tissue, porcine lung organs are easily available from an abattoir. Pigs and men share comparable morphology and physiology and possess similar enzymatic

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Table 2. Comparison of TEER, mannitol and FITC-dextran permeability among different alveolar epithelial cell culture models.

	Human alveolar epithelial cells [34]	Rat alveolar epithelial cells [35]	A549 cells [34]	Calu-3 cells [58]	Caco-2 cells [130]
TEER (Ω cm^2)	2180 ± 62	2450 ± 40	140 – 180 [131]	1000 – 1600	~ 350
Compound			P_{app} (cm/s) × 10^{-8}		
Mannitol				13.5 ± 3	188 ± 11
FD-4	1.71 ± 0.25	1.29 ± 0.06	254 ± 1	0.6 ± 0.05	5.11 ± 0.36
FD-10	1.05 ± 0.11	1.16 ± 0.09	149 ± 11	0.2 ± 0.03	
FD-20	0.80 ± 0.04	1.13 ± 0.17	113 ± 12	0.1 ± 0.01	1.16 ± 0.02
FD-40	0.29 ± 0.03	0.35 ± 0.06	40 ± 6		0.82 ± 0.09
FD-70	0.19 ± 0.07	0.15 ± 0.01	21 ± 4	0.02 ± 0.001	

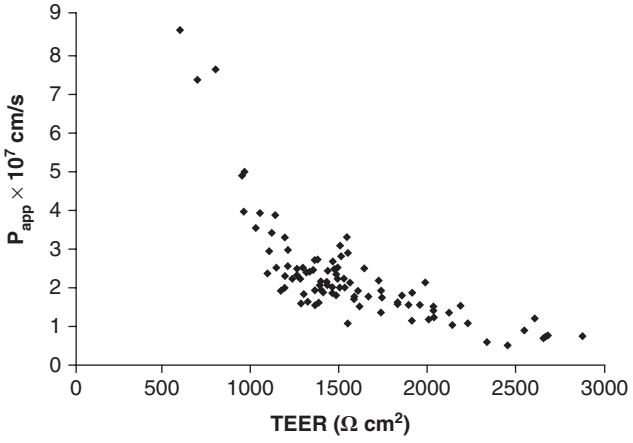

Figure 2. Relationship between TEER across rat alveolar epithelial cell monolayers and the apparent permeability coefficient (P_{app}) for 14C-mannitol.

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TEER: Transepithelial electrical resistance.

equipment [57]. Compared with the rat, the pig provides a larger amount of tissue and reduces the number of animals killed for research purposes. As human and rat cells, type II pneumocytes differentiated into a tight monolayer composed of two cell types resembling type I and type II pneumocytes. The tight junction proteins zonula occludens protein-1, occludin and E-cadherin are expressed in the porcine alveolar epithelial cell monolayer [58]. Although P-glycoprotein is also expressed, its expression was not translated into polarised drug transport [40].

2.2.3 *Air-interface cultures*

Air-interface cultures (AIC) are models that allow aerosol particles to deposit directly onto semi-dry apical cell surface. Drug deposition and dissolution occur in a small volume of cell lining fluid, a situation that mimics more closely deposition on the lung surface *in vivo* [41]. For example, the transport rate of FITC-dextran 4 kDa across Calu-3 cells AIC was 20-fold higher after deposition as a dry powder as compared with a solution because of differences in donor chamber solute concentration [41]. Grainger *et al.* [59] compared Calu-3 cells grown in AIC and in a liquid-covered culture. The AIC showed greater similarity to airways' epithelial morphology, with greater glycoprotein secretion, more pronounced microvilli and the production of a pseudostratified layer of columnar cells, whereas the liquid-covered culture produced a monolayer of cells (**Figure 3**). Nevertheless, Calu-3 cells grown below a liquid showed higher levels of zonula occludens protein-1 than the AIC, which correlated with higher TEER values (1100 Ω cm^2 versus 310 Ω cm^2) [48,59]. Yet, Mathias *et al.* succeeded in reaching 1100 Ω cm^2 TEER using Calu-3 cell AIC [60]. 16HBE14o- cells grown in an AIC did not show clear polar organisation, with weak TEER values (< 130 Ω cm^2). Transport experiments also showed that 16HBE14o- cells in an AIC did not present a functional barrier to drugs [50].

2.2.4 *In vitro/in vivo correlation*

It is important that cell models have similar transport properties as intact lungs in order to obtain results predictable of the *in vivo* response. Therefore, adequate models of lung cell monolayers for studies of drug transport must present solid tight junctions [61]. Mathias *et al.* assessed permeability characteristics of Calu-3 cells to passively and actively transported drugs. They compared these results with absorption across primary cultured rabbit tracheal epithelial cells as well as with published data on *in vivo* absorption from the rat lung [60]. Apparent permeability coefficients in Calu-3 cells correlated well with permeability values obtained in primary culture. Good correlation was also obtained with the rate of drug absorption from the rat lung *in vivo* [60]. Another study assessed dextrans' permeability and found a strong positive correlation between data from layers of Calu-3 cells and from *in vivo* clearance from the canine lung [41]. Manford *et al.* studied the transport of 10 low-molecular-mass drugs across monolayers of 16HBE14o- cells and correlated P_{app} with data published previously in other models [42]. Permeability in 16HBE14o- cells correlated with P_{app} in Caco-2 cells, the most widely used intestinal cell line, with absorption rates in the isolated perfused rat lung and with absorption rate constants from the rat

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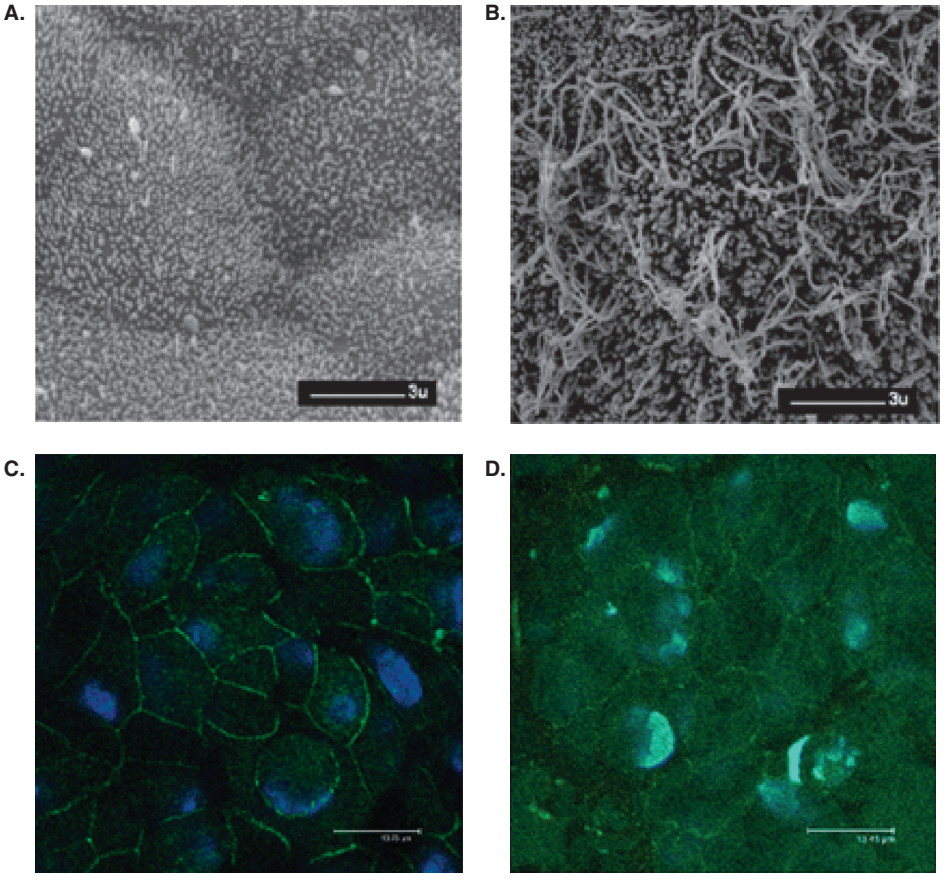

Figure 3. Calu-3 cells grown in a submerged culture (A, C) and in AIC (B, D) at 11 days of culture. A. Images from scanning electron microscopy show small microvilli and well-defined cell–cell boundaries in cell layers in a submerged culture. **B.** Calu-3 cells grown in AIC show heterogeneous population, some microvilli and immature cilia. **C**, **D.** Tight functional protein (ZO-1, green) and nuclei (DAPI, bleu) labelling.
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AIC: Air-interface cultures.

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lung *in vivo* (**Figure 4**) [41]. Tronde *et al.* measured the absolute bioavailability and absorption rate of eight low-molecular-mass drugs from the rat lung *in vivo* [42]. Similarly to Manford *et al.*, they found a correlation between drug absorption rate and P_{app} in Caco-2 cell monolayers. Permeability data of mannitol and FITC-dextrans in various cellular models are presented in **Table 2**. In each model, permeability is inversely related to molecular mass with some sort of plateau in P_{app} at high molecular mass. This supports the idea that the use of common cell models, as Caco-2 cell monolayers, might be sufficient to compare permeability of drugs crossing passively the monolayer, either paracellularly or transcellularly.

Yet, cell culture models present limitations because of their simple organisation with only one cell type and with only one barrier represented, the pulmonary epithelium. Assessment of systemic drug absorption from the lung is limited because the impact of lung regional deposition and clearance mechanisms, such as mucociliary clearance and alveolar macrophages, is missing [61]. This is especially important for macromolecules because they are taken up significantly by alveolar macrophages [30]. However, cell culture models are very useful for the study of transport mechanisms of varying molecules across the epithelial barrier [34,53,55,62,63] as well as for the evaluation of drugs', solvents' or formulations' cytotoxicity [64,65]. Recently, Blank *et al.* developed a triple cell co-culture model composed of a monolayer of A549 or 16HBE14o- cells, human blood monocyte-derived macrophages added on top of the epithelium and human blood monocyte-derived dendritic cells added underneath the membrane in order to study the mechanisms of particle uptake by immune cells in the lungs [66]. Co-culture could be a very useful *in vitro* model to obtain more complete information about drug absorption and clearance mechanisms in the lung.

2.3 *Ex vivo*

The isolated perfused lung (IPL) is an *ex vivo* model. The lung is isolated from rats, guinea-pigs, rabbits or, less frequently, dogs or monkeys. The preparation of the IPL is described briefly hereafter, as it will help in understanding its potentials and limitations. The animal is anaesthetised, a tracheotomy

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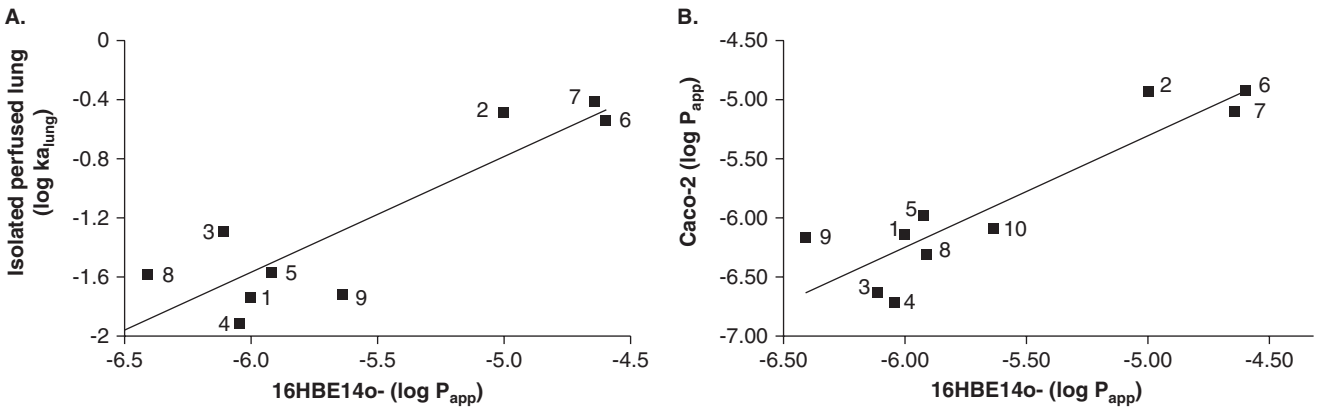

Figure 4. Correlation between apparent permeability in 16HBE14o- cell layers and the apparent absorption rate constant in isolated perfused rat lung (A) and Caco-2 epithelial cell layers (B) for different compounds: (1) atenolol, (2) budesonide, (3) enalapril, (4) enalaprilat, (5) losartan, (6) metoprolol, (7) propranolol, (a-8, b-9) TArPP, (b-8) talinolol and (a-9, b-10) terbutaline.
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is performed and the pulmonary artery and vein are cannulated. The lungs, together with the heart, are surgically removed and suspended by the trachea in a humidified jacketed chamber maintained at 37°C. Perfusion is usually done using a buffer solution, which enters the lung by the pulmonary artery and comes out of it by the pulmonary vein. The buffer solution is then either collected or recirculated. The lung is either left unventilated [67,68] or is ventilated in the artificial thorax chamber, maintained at a pressure below or above the atmospheric pressure [69]. A negative pressure in the artificial thorax is preferable because it represents the *in vivo* situation and negative pressure ventilation decreases oedema formation and atelectasis [70]. Nevertheless, a drawback of negative pressure ventilation is the difficulty of continuous weight recording due to the cycling negative pressure. Uhlig and Heiny presented a weight transducer suitable for assessing weight gain in negative pressure ventilated lungs [71].

Once the IPL model is established, drugs can be administered by the intratracheal route and/or by injection in the perfusate solution in order to simulate a systemic administration [69,72]. Intratracheal delivery can be carried out by nebulisation or instillation of the solution or by dry powder insufflation or inhalation [67,69,72-74]. Lung viability can be checked by visual observation of oedema formation or it can be measured through weight gain in an early stage where there is no visual detection yet. Tronde *et al.* measured lung functions as tidal volume, dynamic compliance and airway conductance as well as perfusion flow at several time points during IPL use, in order to verify lung viability [69].

Sakagami *et al.* compared pulmonary absorption of model molecules in the isolated perfused rat lung and *in vivo* in the rat and modelled kinetic descriptors of absorption from the tracheobronchial and pulmonary regions in the presence of mucociliary escalator [67]. They also modelled pulmonary insulin absorption in the IPL and showed the involvement of metabolism and mucociliary clearance in limiting systemic absorption [75]. Tronde *et al.* investigated the pulmonary absorption of five low-molecular-mass drugs in an IPL from rat and evaluated the IPL-*in vivo* correlation with *in vivo* rat lung absorption results. They observed a strong correlation between IPL and *in vivo* drug absorption half-life [76].

Compared with *in vitro* cell culture models, the IPL is a more complete model as structural integrity and interactions between cells are maintained (alveolar macrophages, epithelial cells, endothelial cells) and the impact of particle size and site of deposition within the lung can be assessed [70]. Compared with *in vivo*, the IPL allows studies on drug absorption from the lung without the influence of the other organs. However, the model does not include absorption from the airways as the tracheobronchial circulation is severed during surgery. This means, for example, that the model largely underestimates pulmonary absorption of small molecules, well absorbed from the airways [67], as well as missing out the possible impact of chemical enhancers on the epithelial barrier in airways [15]. Also, the IPL demands important surgical skills as well as relatively complex technical structure and expensive devices. Another significant limitation of this model is its short viability time (2 – 3 h), where the lung maintains its physiological conditions [75].

2.4 *In vivo*

Before new drugs are delivered to the human lungs, animal studies need to be carried out. The ethics of any animal experiment need to be approved by an Institutional Animal Care and Use Committee. Experiments performed in an animal model can provide information on drug deposition, metabolism, absorption and kinetic profile as well as on drug and formulation tolerability. Non-human primates have very similar anatomy and physiology to humans, but their use is limited because of ethical issues, cost and risk of zoonoses. Therefore, non-human primates are used only in advanced research. By contrast, small rodents (mice, rats and guinea-pigs)

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are common models for initial studies on pulmonary drug delivery because they can be used in large numbers. Mice have been widely used for assessing pulmonary delivery of locally acting drugs: anticancer drugs, antibiotics, immunosuppressive agents and vaccines [5,7,77-81]. The immune system is well characterised in mice and a large number of immunologic reagents is available. Mice have been used less often for assessing pulmonary delivery of systemically acting drugs (e.g., anti-inflammatory drugs [82,83]) because pharmacokinetic studies are not optimally performed in mice. Owing to its small size, one mouse can provide only one blood sample at a time (1 ml whole blood sample is withdrawn by cardiac puncture [80]), and mouse euthanasia must be done at each time point of the plasma drug concentration–time curve [84]. By contrast, pharmacokinetic studies following pulmonary delivery of systemically acting drugs have often been performed in rats, as blood samples at all sampling times can be collected in one rat [85-87]. Guinea-pigs have been widely used as an animal model of allergic asthma and infectious diseases (e.g., tuberculosis) because the airway anatomy and the response to inflammatory stimuli are comparable to the human case [88-90]. Confirmatory testing can be conducted in the rabbit, the dog or the sheep. The dog is a good model for assessing systemic drug delivery by the pulmonary route as well as toxicity [91-93]. The sheep has been used principally as a model of pulmonary hypertension [94,95].

Although studies performed in animal models are fundamental for the evaluation of pulmonary drug delivery, extrapolation of results to humans is not straightforward owing to significant anatomical and physiological differences between species (**Tables 1 and 3**) [39]. Nose anatomy is very different between humans and the other mammals. Humans have a relatively simple and undeveloped nose organisation compared with rodents, which have a complex scroll, or compared with dogs, which present an even more complex turbinate. Humans, dogs and other mammals have their mouth and nose organised in a manner to allow for both nasal and oral breathing. On the contrary, rodents are obligatory nose breathers [96]. These variations of nose complexity between species can cause variable retention of large particles in nasal cavities (elevated in small rodents). The human bronchial branching is symmetric, in contrast to the monopodial branching of non-primate mammals (**Table 1**). Yet, it is noteworthy that, although major differences in lung anatomy exist between humans, large mammals and rodents, these differences do not translate into significant differences in optimal size of aerosol particles for alveolar deposition (**Table 3**).

The different mammals do not appear to present similar mucociliary clearance and alveolar macrophage morphometry. In large mammals, the rate of mucus clearance in millimetres per minute is high compared with small rodents (**Table 3**). However, large mammals also have longer airways than small rodents and thereby, globally, the bronchial clearance of inhaled particles is relatively slow in humans (> 24 h). By contrast, bronchial clearance of particles is relatively fast and early in rats and mice [39,97]. The number of macrophages per alveolus and the alveolar macrophage volume are greater in human and canine lungs than in small rodents' lungs (**Table 1**) [98]. For example, alveolar macrophages are twofold and threefold bigger in humans than in rats and mice, respectively [98]. These variations add up to the difficulty of the extrapolation of the results from non-human mammals to humans and underline that great caution is needed in the extrapolation.

There are several methodologies available for pulmonary administration of drugs, each of them with specific advantages and limitations, which are discussed below. Drugs can be administered by passive inhalation or they can be administered directly to the lung in both a liquid or powder form.

2.4.1 *Passive inhalation*

During passive inhalation of aerosolised drugs, animals are kept awake and allowed to breathe normally. Aerosolised drugs are delivered using an aerosolisation chamber in whole body, head-only or nose-only exposure systems. The devices most frequently used for generating aerosols are nebulisers [77,79,99]. However, aerosolisation of dry powders has also been reported in several recent studies [82,100,101].

Passive inhalation is principally used in the mouse and less frequently in larger animals (rat, guinea-pig, dog). This method is more representative of drug delivery to the human lungs than intratracheal instillation of large volumes of liquids. However, significant losses of the drug dose occur in the reservoir and tubing of the aerosol generator, in the delivery accessories (aerosolisation chamber) to the animal and during animal expiration. This results in a low and poorly controlled drug dose delivered to the animal (~ 0.1%) and actually delivered to the lungs (< 0.01%) [101-103]. Therefore, passive inhalation is not the method of choice for expensive drugs, for pharmacokinetic and vaccination studies because the dose delivered is not known with accuracy and systemic absorption or immune responses could originate from other mucosa (e.g. nasal).

In general, the drug dose delivered to the animal (whole body dose) is estimated using the following equation:

$$\text{Dose} = \frac{\text{Aerosol concentration} \times \text{Repiratory minute volume} \times \text{Exposure time}}{\text{Body weight}}$$

The drug concentration in the aerosol is determined by sampling the test atmosphere and quantifying the drug in the sample. The respiratory minute volume can be estimated based on values reported in the literature [78,79,101]. The size distribution of aerosol particles can be measured in order to verify its adequacy for pulmonary delivery [77].

2.4.1.1 *Whole body exposure system*

In whole body aerosol exposure system, animals are placed in a sealed plastic box that is connected to a nebuliser or a generator of dry powder aerosol [82,104,105]. Although this system allows a less stressful pulmonary drug administration to an important number of animals, there is potential drug

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Table 3. Comparative respiratory parameters in mammals [127,134,135].

Parameters	Human (70 kg)	Rhesus monkey (5 kg)	Beagle dog (10 – 15 kg)	Rabbit (2.5 – 3.5 kg)	Guinea-pig (0.4 kg)	Rat (0.25 – 0.35 kg)	Mouse (0.02 – 0.04 kg)
Nose and/or mouth breather	Nose/mouth breather	Nose/mouth breather	Nose/mouth breather	Nose breather	Nose breather	Nose breather	Nose breather
Respiratory rate (min^{-1})	12	38	23	51	90	85	163
Tidal volume (ml)	400 – 616	20 – 21.2	11.4 – 16.6	15.8	1.72 – 1.75	0.87 – 2.08	0.15 – 0.18
Total ventilation (l/min)	7.98	1.67	1.5	0.8		0.12	1.025
Mucus clearance (mm/min)	3.6 – 21.5	-	7.5 – 21.6	3.2	2.7	1.9 – 5.9	-
Particles size range for alveolar deposition (µm)	1 – 5 [8]	-	1 – 3 [92]	-	-	3.5 [136]	3 [102]

absorption across the skin after deposition on the animal fur, from the nasal mucosa and from the gastrointestinal tract [102]. The alleviation of stress to the animal is an advantage because stress has been linked to physiological changes that can alter experimental data. For example, psychological stress can downregulate cellular immune responses and modify gene expression [106,107].

2.4.1.2 *Head-only or nose-only exposure systems*

In the head-only or nose-only exposure systems, the animal is attached to the exposure chamber and only the head or the nose is in contact with the aerosol. The systems can be designed for delivering drugs to one or to several animals. Compared with the whole body exposure system, the head-only or nose-only exposure systems offer several advantages. Skin exposure to the drug and its uptake by the transdermal route are avoided. The low volume of the aerosolisation chamber reduces the amount of drug needed to generate the aerosol. Potential drug reactivity with excreta is avoided. Variable durations of animal exposure are possible in one single test. Air exhaled by the animal can be thrown out from the aerosolisation chamber. This is an advantage because the high humidity of exhaled air can affect the stability of aerosolised drugs or modify the particle aerodynamic behaviour. In addition, increased carbon dioxide in the inhaled air can stimulate ventilation and modify acid–base status [108]. Head-only or nose-only aerosol exposure systems are commercially available but there are some 'home-made' designed aerosol boxes described in the literature [100,109].

2.4.2 *Direct intratracheal administration*

Intratracheal administration of drugs is the favourite method when a precise control of the dose is needed because drugs are administered directly in the trachea. In addition this mode of delivery circumvents nasal and oropharyngeal deposition. Direct intratracheal administration is the technique used most of the time in large animal models such as the rabbit, sheep and dog. It is also frequently used in the guinea-pig and rat, but it is less frequently used in mice. In rodents, the following procedure can be followed. After anaesthesia, the animal is laid in a supine position, attached by its superior incisors to a board and tilted at an angle of 45 degrees [7,15]. The mouth is kept open and it is possible to see the vocal cords and trachea with the help of a laryngoscope (e.g., the small animal laryngoscope from Penn Century, Philadelphia, PA). The administration is performed by inserting a cannula in the trachea, between the vocal cords [15]. It is also possible to perform intratracheal administration using a surgical procedure to expose the trachea, and the cannula is then inserted between two cartilaginous rings [110]. However, because of its invasiveness, this method should not be preferred.

Drug solution can be delivered into the trachea as a liquid bolus by intratracheal instillation, as a coarse spray by using a spray-instillator, or as an aerosol generated by a nebuliser [94,111]. Dry powders can be delivered intratracheally using a powder-insufflator (Dry Powder Insufflator, Penn Century, Philadelphia, PA) or by generating a powder aerosol [91,92].

Although intratracheal administration is a simple method of pulmonary drug delivery, small changes in the method can lead to significant differences in site of drug deposition within the lung and, thereby, in systemic drug absorption. For example, Codrons *et al*. compared three methods of intratracheal instillation in the rat and they showed that each method resulted in a different bioavailability of parathyroid hormone 1 – 34 owing to differences in regional deposition within the lung. Deposition of the solution in the trachea, central and peripheral lobe sections was assessed after tissue grinding using albumin as a slowly diffusing marker. The use of a simple microsyringe led to the deepest administration within the lung and to the highest bioavailability when the instillation was followed by the administration of a 3 ml air bolus. A spray-instillator, producing 25 – 30 µm solution

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droplets, led to more central deposition and lower bioavailability [15]. Minne *et al.* demonstrated that the technique of instillation could be optimised in order to target the nasal passages, the upper or central airways or the deep lung in mice as well. Variations in the technique comprised the route of instillation (nose or mouth), the volume of solution, the position of the mouse (tilted or not) and the possible insufflation of a 200 µl air bolus following instillation [7].

Advantages of intratracheal administration of drugs include the perfect control of the drug dose delivered, the absence of drug losses in the instrumentation (except for liquid and powder aerosols), the bypassing of nasal passages and the possible targeting of different regions within the respiratory tract. However, this method of administration is not representative of the natural inhalation process as the solution is forced into the airways and the animal is under anaesthesia. It is not recommended for repeated administrations as each insertion of a cannula into the trachea generates slight but significant inflammation. A limitation peculiar to intratracheal instillation comprises the administration of large volumes of solutions as compared with the volume of the epithelium lining fluid (ELF; **Table 1**). For example, the rat ELF volume is 45 – 55 µl [112], whereas the volume of solution instilled is ~ 100 µl in general, almost twice the ELF volume. The administration of large volumes of liquids can cause changes in lung physiology and decrease drug concentrations locally.

2.4.3 *Intranasal administration*

Intranasal administration is mostly known for local drug delivery to the nasal mucosa but it can also be used for intrapulmonary drug administration in mice [113-115]. Intranasal administration is performed on the anaesthetised mouse kept in a vertical position. With the help of a micropipette, the solution is deposited on a nostril and is simply aspirated in respiratory airways during breathing. Minne *et al.* showed that the use of a small volume of solution (5 µl per nostril, 10 µl total) restricted drug administration to the nasal cavity but that the use of a larger volume of solution (25 µl per nostril, 50 µl total) allowed a deeper administration to be reached in lung upper airways [7]. This method is technically easier than intratracheal administration but it presents the limitation of administering drugs mainly to upper respiratory airways.

2.4.4 *End points*

Several end points can be assessed following pulmonary drug delivery to the animal *in vivo*. They can be categorised into local concentrations and effects, systemic absorption and effects, toxicity assessment and non-invasive imaging.

Drug concentrations can be measured in lung tissue or in the ELF. Measurement in lung tissue requires lung resection, homogenisation and drug extraction using an adequate solvent for the drug [77,109]. Drug concentrations in the ELF are estimated by using bronchoalveolar lavage (BAL). BAL consists in injecting a saline solution into the lung followed by its aspiration. In small animals, BAL is performed on whole lung following animal euthanasia [7]. In large animals, BAL is performed on a lung section after animal sedation [116]. Local effects can be quantified in lung homogenates and visualised on histology slides [99]. This is the case, for example, for the protection conferred by antibiotics in lung infection where bacterial burden is measured in lung homogenates and histopathology analysis is performed on lung tissue [90].

Systemic drug absorption following pulmonary administration is classically assessed by pharmacokinetic studies. Pharmacokinetics are performed not only for systemically acting drugs, but also for locally acting drugs, as a way to assess lung deposition efficacy of formulations [80,90]. Implantation of catheters in jugular (or femoral) veins is highly recommended for blood sampling in rats and guinea-pigs because blood samples from the tail vein can largely underestimate (by one order of magnitude) actual plasma drug concentrations. In mice, whole blood is generally withdrawn by cardiac or orbital puncture at each time point of the plasma drug concentration–time curve. In rabbits, the central ear artery or marginal ear veins are used for blood sampling. Useful pharmacokinetic parameters include the area under the plasma concentration–time curve (AUC), the maximal plasma concentration (C_{max}) and the time to peak (t_{max}). Absolute and relative bioavailability values can be obtained by conducting further pharmacokinetic studies following other routes of drug delivery (injection or oral administration). Pharmacodynamics can also be assessed following pulmonary drug delivery. Glycaemia, for example, is easily measured in blood samples following administration of insulin [93]. Similarly, calcaemia can be measured following calcitonin administration [117].

A major concern of pulmonary drug delivery is the potential toxicity caused by drug administration. To evaluate safety, several markers of pulmonary inflammation can be assessed in BAL fluid and histology slides of the lung tissue can be examined for structural alterations [5,85,118]. Lactate dehydrogenase activity and protein levels are biochemical markers of inflammation that are measured in BAL supernatants and indicate tissue injury and alteration of epithelium permeability, respectively. The cell pellet from the BAL can provide the total number of cells withdrawn from the lung airspaces and the cell differential counting can indicate inflammation by the presence of neutrophils. For further analysis, inflammatory cytokines such as TNF-α can be monitored in BAL supernatants [5].

Recent developments in techniques of radionuclide imaging have enabled quantification of whole as well as regional lung deposition in large and small animal models [119,120]. Gamma scintigraphy is a two-dimensional imaging method with a spatial resolution of 10 mm. The radionuclide ^{99m}Tc has been used for studies on pulmonary drug delivery. The energy of the gamma rays emitted by ^{99m}Tc enables high-quality images to be obtained in humans, monkeys, dogs and rabbits [111,119,121]. Drug particles are almost always physically associated with ^{99m}Tc radiotracers, without chemical bound [119]. A gamma

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camera is a large radiation gamma detector containing a crystal traditionally composed of sodium iodide [119]. Single photon emission computed tomography (SPECT) is a three-dimensional extension of gamma scintigraphy. It uses gamma cameras with two or three heads that can rotate around a subject. Data from multiple angles are acquired as the gamma camera heads rotate and these are used to reconstruct the original radionuclide distribution in the lungs [119]. Special design of the gamma camera in SPECT has improved spatial resolution and has permitted imaging of the lungs in rodents [120]. Positron emission tomography (PET) can also be used to image the pulmonary deposition of drugs in three dimensions. As the drug itself can be labelled, quantification of drug clearance is possible. PET uses radionuclides that decay by positron emission, including ^{11}C, ^{13}N and ^{15}O. The emission of a positron is followed very rapidly by its annihilation as it encounters an electron. This produces two photons that are detected [119]. The spatial resolution of PET has recently been increased from 5 to 1 mm through the development of high-resolution detectors and this resolution allows lung imaging in rodents [119,120]. Although radionuclide imaging techniques can provide informative data, drawbacks include the cost of the equipment and the technical challenge of drug radiolabelling.

3. Conclusion

This article has reviewed the models of the normal healthy lungs and has shown that each provides important and complementary information for the development of inhaled drug products. Disease models are beyond the scope of this article and information on disease models can be found in other articles [122-125].

Although deposition data obtained from cascade impactors *in vitro* are not fully predictive of aerosol deposition in the human lung, the analysis provides key information as the mass median aerodynamic diameter of the aerosol and allows control of the quality of the pharmaceutical product over its shelf-life. Cell culture models *in vitro* are perfectly adapted for comparing the relative transport rates across epithelia of compounds with varying physicochemical characteristics. They also permit analysis of mechanisms of transport of macromolecules and actively transported drugs across epithelial barriers. Mechanisms of action of chemical enhancers can be delineated in cell cultures. Yet these *in vitro* models are not predictive of the final bioavailability of the pulmonary route for a particular compound because they miss the other barriers to systemic absorption. Cell culture models are also very useful for assessing pulmonary toxicity of new drugs and new formulations. The *ex vivo* isolated perfused lung provides quantitative and mechanistic data on systemic drug absorption following pulmonary delivery. Yet the loss of the tracheobronchial circulation precludes assessment of its contribution to total systemic absorption from the lung. In this regard, the *ex vivo* model can provide very complementary data to studies carried out *in vivo*. Finally, *in vivo* models provide the most global and quantitative view on systemic drug absorption from the lung. Also, it is the only model capable of providing screening data on therapeutic drug efficacy as well as of assessing efficacy of pulmonary vaccination, where the mouse is the reference model. Although studies on lung clearance mechanisms can be carried out *in vivo*, detailed mechanisms of drug transport across the lung epithelium cannot be delineated.

4. Expert opinion

Extrapolation of *in vivo* data in animals to the clinical situation is not straightforward, particularly from in vivo data in small rodents. Many experimental factors are modified in animal studies, which already makes comparison between studies difficult. For example, previous investigations on drug absorption from animal lungs have reported bioavailability values that can vary by one order of magnitude between studies, for the same drug and in the same animal species [15,126]. These variations can principally be explained by the varying methods of administration used. The methods used to administer drugs to the lungs involve different drug losses in the delivery device (highly significant in passive inhalation), different drug losses in the animal (high drug deposition in the nose following passive inhalation) and different site of drug deposition within the respiratory tract. In addition, the site of drug deposition within the respiratory tract is usually not determined in pulmonary drug delivery studies although it has a major impact on systemic drug absorption [15]. All these factors confuse comparison between animal studies. Yet correlation between systemic absorption of varying drugs in animals and in humans can be obtained, provided the same animal model, the same method of drug delivery and the same drug formulation are used. Of course, the best guarantee that an identical method of drug delivery is used in the animal is provided when data are generated in the same laboratory.

When comparing systemic drug absorption from different drug formulations *in vivo*, it is also not straightforward to obtain an identical site of drug deposition within the respiratory tract, especially when comparing a liquid formulation with a dry powder. Therefore, comparison of varying drug formulations and the study of the impact of excipients on drug transepithelial transport are best carried out *in vitro* in cell culture models and AIC is the most representative *in vitro* model of the epithelial barrier *in vivo*.

Declaration of interest

This work was supported by the Réseaux programme of the Walloon Region (grant n° 415695; Belgium). Rita Vanbever is Maître de Recherches of the Fonds de la Recherche Scientifique (Belgium).

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