

***Title:***

***In Vivo* Animal models for Drug Delivery Across the Lung Mucosal Barrier**

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**Abstract:**

Over recent years the research focus within the field of respiratory drug delivery has broadened to include a wide range of potential applications for inhalation by delivering drugs not just onto the lung mucosa but across it. The range of drugs being assessed is broad and includes both current and novel therapies and there are a growing number of additives that appear capable of enhancing systemic absorption. Comprehensive characterisation of drug delivery to the lungs is a complex task involving determination of delivered, deposited and for systemically targeted drugs absorbed dose and therefore difficult to simulate *in vitro*, thus *in vivo* whole animal models are still key in inhaled drug development. Because of the anatomical complexities and interspecies differences in the lungs appropriate choice of species and drug delivery method is vital during study design. New delivery devices designed specifically for animal studies and more sophisticated methods to determine drug deposition and absorption after inhalation are improving the information now derived from these studies.

**Keywords:** systemic drug delivery, respiratory delivery, inhalation, aerosol, pharmacokinetics, toxicity, drug deposition, drug absorption

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## **1. Introduction**

In modern medicine **drug** inhalation has primarily been used as a modality for treating local respiratory diseases and over 500 million people now carry the **classical** metred dose inhaler with them on a daily basis [1]. Over the last 20 years the research focus within the field of respiratory drug delivery has broadened to include a wider range of potential applications for inhalation by delivering drugs not just onto the airway but across it [2,3]. This change in thinking was catalysed by a number of factors including the emergence of biotherapeutics with their associated delivery issues and a greater understanding of the absorption properties of the lungs. The physiology and anatomy of the lungs offer a unique portal for drug delivery. Its' large surface area, decreased metabolic capacity (relative to the gastrointestinal tract), a relatively thin alveolar epithelium in the lower airways and rich blood supply have been found to facilitate rapid drug absorption and **adequate bioavailabilities** via the lungs [4,5]. Inhalation has the added benefit of being a non-invasive route of delivery, particularly appealing for all the biomolecules currently restricted to parenteral use [6,7,5].

### **1.1 The human lungs as organs of absorption**

The human lungs are composed of a series of subdividing airways with airway calibre diminishing from the trachea, through the bronchi and bronchioles (known as the airways) and finally to the alveolar ducts and sacs, the site of gas exchange [8]. There are significant differences between the cells located in the airway and the alveolar epithelium, with the airways composed of pseudostratified columnar epithelium containing mucus and cilia cells that constitute the mucociliary clearance mechanism of

the lungs. In the alveoli the columnar epithelial cells are replaced primarily by broad and thin Type I cells alongside their progenitor Type II cells that also produce lung surfactant. In this region too are found the alveolar macrophages. The thickness of epithelium decreases from 50-60 $\mu$ m in the upper airway to 0.2 $\mu$ m in the alveolar region. In terms of volume the conducting airways (trachea, bronchi and bronchioles) constitute approximately 150ml while the respiratory zone of the lungs comprises some 2.5-3 litres in volume. The alveolar region makes up the vast proportion of the overall surface area of the lungs with an alveolar surface area of more than 100m<sup>2</sup> compared to only a few metres square in the airways[8]. The large surface area of the lungs and the highly perfused nature of the organ mean that conditions are well suited for drug uptake into the systemic circulation. The alveolar regions of the lower lungs appear to be the optimum site for systemic drug absorption of many drugs including the majority of peptides and proteins[6], while small, hydrophobic molecules are absorbed rapidly by diffusion throughout the lungs[6]. The routes of drug absorption across the airway epithelium can include passive and active transport mechanisms involving paracellular and transcellular transport, pore formation, vesicular transport, and drainage into the lymphatics depending on the drug and site of absorption [912].

The lung does however present barriers to systemic absorption of drugs. The very branched architecture of the respiratory tract means that efficient delivery of a drug to the lower lungs, where the majority of drugs are best absorbed, is difficult. Efficient delivery to the lower lungs after inhalation is essential if adequate absorption to achieve the desired systemic concentration is to be facilitated. Pharmaceutical inhalation technology

dictates that particles with an aerodynamic diameter between 1-5 $\mu$ m are deposited in the lower lungs, with larger particles impacting in the throat and smaller particles being exhaled. In reality there are a complex range of critical factors for efficient deposition of particles in the lungs including the size, density, shape, velocity, charge, hydroscopicity and surface properties of the particles as well as the breathing pattern of the patient (e.g. ventilation volume, flow rate, end-inspiratory breathholding)[13] and the state of the respiratory system including the patients age, sex, anatomy, lung volume and disease state[14]. Recent advances in inhaler/nebuliser device design [1518] and therapeutic aerosol bioengineering (TAB) [19] means that improved delivery efficiencies can now be achieved in the context of optimised formulations and breathing patterns for a given device. This is of particular importance for systemic delivery of drug molecules when absorption is site-specific and also for expensive biotherapeutics, where poor delivery efficiency would make therapy prohibitively expensive.

The alveolar epithelial cells and not the underlying endothelial cells are the major barrier to transport [20]. Though the alveolar epithelium is highly permeable to water, gases, and lipophilic substances, the permeability of large, hydrophilic substances, such as proteins is limited [21]. The non-absorptive clearance processes of the lungs are another hurdle to systemic delivery and can have a major impact on the amount of active drug available for transport across the mucosa. The role of mucociliary clearance in the tracheobronchial tree is to remove unwanted irritant particles from the lung, therefore insoluble drugs and/or insoluble delivery systems deposited in the upper airways are rapidly cleared [22]. Insoluble drugs and carriers deposited in the alveolar region will tend to be taken up by

the macrophages, which has recently been demonstrated to pose a primary barrier to pulmonary absorption of macromolecules [23]. They can dispose of particles either by transporting them along the alveolar surface to the mucociliary escalator, by translocation to the tracheobronchial lymph or by internal enzymatic degradation [24]. In the case of protein and peptide drugs this can mean rapid degradation in the lysosomal system. Drug metabolising enzymes are present in much lower concentrations in the lungs [25] than in the gastrointestinal tract and the degree of drug metabolism appears to be drug-type dependent with small peptides being hydrolysed rapidly while for small molecules metabolism is minimal. A certain degree of metabolic clearance of insulin has been demonstrated in recent kinetic studies which would appear to impact on its <10% bioavailability in both human and animals alongside mucociliary clearance [26].

### ***1.2 Drug candidates for systemic delivery via the lungs***

The majority of licensed topical drugs for inhalation have to-date been small molecules ( $\beta$ -agonists, steroids, antibiotics etc.). While the potential of inhalation for delivery of biotherapeutics has gotten most of the press attention it is important not to overlook the advantages of this route for small molecule drug delivery to the systemic circulation. A number of small drug molecules appear to be viable candidates for systemic delivery via the lungs (Table 1). The rate of absorption of small molecules from the lungs is second only to intravenous delivery [5] and bioavailability tends to be higher than oral bioavailability. The main reasons for this is the decreased metabolic capacity in the lungs and the lower levels of drug efflux transporters expressed on the airway cells compared to the gastrointestinal epithelium. Just as for oral absorption the nature of the molecule

affects the rate of absorption with hydrophobic molecules displaying extremely rapid absorption in seconds to minutes while hydrophilic molecules tend to have a slightly longer half-life of about 60 minutes [5]. Because of the very rapid onset of action of small molecules after inhalation it is thought that inhalation of the relevant small molecule could potentially be used to treat conditions such as seizures, arrhythmias and hypertensive crisis where speed is of the essence.

Pulmonary peptide and protein delivery appears to be an increasingly viable option for their systemic delivery [7,27] with bioavailabilities surpassing routes such as transdermal, nasal and buccal. Two proteins for inhalation are currently available on the market DNase (locally acting mucolytic for Cystic fibrosis) and now to the delight and relief of many working in the field, insulin. A wide range of other proteins and peptides for systemic delivery via the lungs are being investigated, including calcitonin, growth hormone, immunoglobulins, and granulocyte colony-stimulating factor (G-CSF) to name a few (Table 1) The advantages of this route for macromolecular delivery are well documented and recently reviewed [7,28,5,29,30], with the molecular weight of a given macromolecular drug candidate impacting significantly on the rate of absorption [31,32] and metabolism[7,5]. Despite its perceived potential, progress to market in the area of systemic delivery of proteins has been slow to-date due to a number of confounding factors including rapid clearance, instability [33], risks of immunogenicity [33] and long-term toxicity, dosing issues[28] and limited additives licensed for inhalation. These issues have been covered in some detail in recent reviews [29,30].



### **1.3 Strategies to enhance systemic drug delivery via the lungs**

A number of different approaches to improve and/or control systemic drug delivery via the lungs and overcome some of the barriers previously discussed are being investigated. These include improved delivery efficiency to the lower lungs, molecular engineering of the drug molecule itself and/or addition of various excipients to enhance absorption and/or retain the drug at the site of absorption (Table 2).

To facilitate the delivery of expensive biotherapeutics as well as enhance systemic drug delivery a range of new generation devices have been engineered that produce much higher respirable doses. These include a new generation of liquid-spray devices [34,35] that generate an aerosol mechanically, vibrating mesh technologies [17] and dry powder inhalers that produce inhalable aerosols independent of the patients' inspiratory flow rate and volume [36,18].

Altering the molecular structure of a drug can be used to alter its kinetics in the lungs with the obvious caveat of preserving the activity. Studies indicate that cationic and hydrophobic small molecules have a longer half-life in the lungs [27] and for small peptides the degradation rate in the lungs can be decreased by cyclising the peptide or modifying the C or N terminal ends to block peptidase attack. Modifying drugs, in particular peptides and proteins, with polyethylene glycol (PEG) [37,5] groups also increases retention in the lungs thereby increasing bioavailability after inhalation [38].

Various formulation approaches have been tried such as the use of penetration enhancers (decreasing the epithelial barrier) and enzyme inhibitors (decreasing the proteolytic barrier) [3941]. The application of both approaches has been limited, however, due to safety concerns but some success has been achieved with low molecular weight amino acid analogues[42]. Ligands that specifically trigger an active uptake process in the alveolar cells may be preferable from a safety perspective to generalized permeation enhancement that allows the non-specific passage of other particles across the airways. [11,43].

Bioengineering of therapeutic aerosols using carriers such as liposomes and microspheres have been assessed as a strategy to improve systemic absorption of drugs via the lungs [44]. Both liposomes [45,46] and microspheres [4749] have been used to enhance the systemic effect of insulin after inhalation. The concentration, charge and acyl chain length of the phospholipids components of liposomes are critical to their ability to enhance systemic delivery [46]. The beneficial effects of microspheres on systemic absorption are attributed to the sustained release of the insulin from the polymeric carriers [50]. Other strategies to decrease clearance and prolong mucosal drug exposure have employed bioadhesive polymers e.g. HPC [51].

#### **1.4 Ex-vivo models of drug absorption across the lungs**

While cell cultures are an increasingly important tool for preliminary studies of drug transport and drug transport mechanisms across airway cells, the potential permeation enhancing effects of excipients and for a preliminary assessment of the potential toxicity

of a drug/drug delivery system [52] there are limitations to their utility in absorption kinetic studies and in order to overcome some of these limitations *ex vivo* models of lung absorption have been developed. One of the most commonly used *ex vivo* methods is the isolated perfused lung model (IPRL). The lungs are isolated from the body of an animal (generally rats or guinea pigs) and housed in an artificial system under defined experimental conditions [53,54]. Peristaltic pumps carry perfusate (autologous blood or a buffered artificial medium equilibrated with a mixture of oxygen and carbon dioxide) to and from the organ which is housed at 37°C and the system can be maintained at a specific pressure. Drugs/delivery systems are administered via the tracheal port. Sampling of the perfusate allows the rate of absorption of the drug to be determined. This *ex vivo* model offers a number of advantages over isolated cell culture models because of the maintenance of the structure and functionality of the tissue.

Limitations of these models include short viable periods of 2–3 hours, the likely absence of tracheobronchial circulation and the high level of training and expertise required to set-up the IPRL. In spite of this *ex vivo* models have been shown to be kinetically predictive of *in vivo* with respect to certain molecules, most notably macromolecules. There appears to be a less certain correlation for small molecules. The reason for this would appear to be the absence of the tracheo bronchial circulation in this model. While large molecules are generally not absorbed *in vivo* across the thick tracheobronchial epithelia small molecules are, therefore the lack of circulation in this region in IPRL makes modeling their absorption difficult. Isolated tracheal tissues used *ex vivo* have been used to characterise tracheobronchial airway absorption when relevant.

One of the advantages of these *ex vivo* models for the purposes of modeling *in vivo* absorption and clearance is of course the presence of a whole organ with the full range of cell types, this can however make assessing the mechanism of absorption more difficult. Nevertheless recent improvements in kinetic profiling have enabled the kinetics of not only passive and/or active absorption to be determined but also those of the kinetics of drug clearance from the lungs (including phagocytosis, mucociliary clearance and metabolism). Studies also indicate that the appearance of test molecules in the perfusate correlates well with the depth of aerosol penetration. Unfortunately because of the complexity and cost of the set-up and the limited throughput of this model its' use to-date has been limited.

Therefore in order to properly assess the feasibility of systemic drug delivery after inhalation and the effect of new device/molecular engineering/particle bioengineering strategies to improve absorption animal models are vital. The anatomical and physiological differences between species have to be taken into careful consideration as well as the method of delivery of the drugs.

## **2. Animal Models**

Ideally, the evaluation of factors influencing the delivery of drugs across the airways should be conducted in healthy or diseased humans. Animal models, however, allow manipulation of the different variables involved that otherwise may not be easy or feasible in human subjects. A large number of studies have employed rodents, but other

animal models including rabbits, dogs, sheep, monkeys and non-human primates have also been used. Recently, Sakagami [55] reviewed the in vivo, in vitro and ex-vivo models available to assess pulmonary absorption and disposition of inhaled therapeutics for systemic delivery. This article gives an excellent perspective on the available animal models and techniques. The present section extends to anatomical, physiological and technical considerations required for the selection of the species and method of aerosol administration involved in a particular study.

Initial studies in virtually every area of pharmaceutical and pharmacological research have been performed in small rodents such as mice, rats and guinea pigs because terminal procedures can be easily conducted and large numbers of these animals can be used for statistical validity. Rats and guinea pigs are frequently used for the study of drug delivery to the lungs because a variety of dosing techniques that require a small amount of the drug can be employed and they are good models for a number of respiratory disease states. Studies of the comparative biology of the guinea pig have revealed that the physiology of their pulmonary tract is quite similar to that of humans, particularly the response of the lung to inflammatory stimuli as well as the dermal response to both acute and chronic inflammatory mediators [56,57]. Therefore guinea pigs are regarded as a good model of bronchoconstriction/ bronchodilation in the evaluation of drugs used to treat asthma. Hormonally and immunologically guinea pigs are more like humans than other rodents; thus they are often used to model human infectious diseases [58]. Although rats are not as similar to humans they have been used to study a range of diseases such as emphysema [59], influenza [60] and pulmonary fibrosis [61]. Other systemic diseases

such as diabetes can be induced in rats by administration of streptozocin [62]. Delivery of large doses of aerosol to mice is difficult to achieve in short periods of time and the amount of biological fluids that can be collected is small. Nevertheless, mice have been used to investigate drug delivery in disease states such as cystic fibrosis [63] and lung cancer [64].

The use of larger species such as rabbits, dogs, sheep and primates is considered when the study design is more complex, requiring more sophisticated maneuvers, administration of larger doses of drug or collection of larger amounts of body fluids for longer periods of time is required. However, regulatory agencies and cost may limit the number of animals employed in these studies, which in turn may influence the statistical analysis of the results. The dog model has been utilized to study the delivery of insulin [65] or the treatment of some allergies [66]; monkeys and non-human primates have been employed to assess vaccine delivery [67] or drugs to treat cystic fibrosis [68], where sheep are considered a good model of lung injury [69].

## **2.1 Rational model selection**

Although many species are suitable for studies of disposition of drugs, in and from the lungs, the organization of cells and tissues in the lung is an important consideration in the selection of an animal model when the results are to be extrapolated to humans. This is crucial when the quantitative description of airways may be used in mathematical models for predicting efficiencies of deposition and clearance of inhaled drugs. Comparisons of

the morphologic characteristics of the lungs of many mammalian species have been reported [70] and key differences in individual anatomical parts are discussed below.

Some of the anatomical characteristics of the lungs for several species are compared to those of the human lungs in Table 3 as revealed on replica casts [70]. In overall shape, the human lung tends to be more spherical than the lungs of other species. The significance of overall shape is seen in the relatively symmetrical airway branching scheme at essentially all levels in the human compared with the long tapering monopodial airways with small lateral branches characteristic of all the other species. Tracheal length-to-diameter (L/D) ratios differ considerably among mammals as do the branching angles. The guinea pig and rabbit have relatively long tracheas; dog, monkey, rat and hamster are intermediate, while human is relatively short as compared to other mammals. In terms of the number of lobes, the left human lung is divided into two lobes, the superior and inferior, and the right lung is divided into three by the addition of a middle lobe. The right lungs from laboratory mammals including non-human primates are divided into four lobes. The left lungs from mice and rats are not divided but those from larger mammals such as guinea pigs and rabbits are divided [71]. Several studies have indicated that the variations in airway branching patterns and the consequent effects on airflow across species can contribute to differences in regional deposition in the lungs[72,73] but this must be seen in the context of other contributory factors e.g. differences in breathing patterns, obligate nose-breathing and the physics of the aerosols delivered[74]. Overall it would appear that the airway anatomy and oral breathing of humans tends to lead to a greater amount of upper bronchial airway particle deposition

and to greater deposition on localized surfaces near airway bifurcations compared to smaller mammals[75].

Table 4 shows the morphometric characteristics of cells in the alveolar region of the lungs of rats, dogs, baboons and humans [76] . Compared with the other species, human lungs contain greater number of macrophages. Significant interspecies differences in diameter, volume, number and functionality have been reported between human, monkey and rats/hamsters alveolar macrophages [7780], which may have implications for effective clearance. The thickness of the interstitium and the pulmonary capillary endothelium of the human lungs were also significantly greater. In addition, turnover times for several cell populations in the lung are significantly different between species [81].

For respiratory mechanics, the same expressions are used for lung volumes in man and in other mammals, but the mechanics that define most of them are different [82]. Total lung capacity and residual volume are maximum and minimum volumes, achieved in co-operative humans by voluntary maximum effort but in animals by application of external pressures whose magnitude and timing are not standardized. Marked variations are also present in the lung volume and breathing frequency of different species (Table 5).

## **2.2. Comparative responses in the respiratory tract.**

In all types of inhalation studies, at least three aspects of exposure to aerosolized drugs should be considered: deposition, clearance and the magnitude and type of the biological



response. Stauffer used dimensional analysis to predict that the probability of deposition of inhaled aerosols should be the same for different animals in the case of sedimentation or turbulence-dominated deposition, but should scale as (body weight)<sup>0.1</sup> for a diffusion dominated deposition [83]. Conversely, McMahon et al. suggested that diffusion dominated deposition is independent of body weight [84]. Studies in six different species showed that when the total amount of aerosol deposited was divided by the animal's body weight, the smaller animals had received more particles/g than the larger ones, but the collection efficiencies (fraction of inhaled aerosol deposited) for both the lungs alone and the lung, nose pharynx and airways combined were substantially independent of body size.

The different rates of clearance among species can also influence retention and, therefore, the total dose to the lungs. Brain et al. demonstrated that *in situ* pulmonary macrophages of hamster cleared insoluble gold particles faster than rats, rabbits and mice [85]. Different species have also demonstrated varying degrees of efficiency in the bactericidal activity of alveolar macrophages [86]. Rabbit alveolar macrophages avidly ingest and kill *S. aureus*, whereas alveolar macrophages from rats ingest but are rather poor at killing the same organisms. Nguyen et al also reported significant differences in phagocytosis and killing by pulmonary macrophages from humans, rabbits, rats and hamsters [87]. The relative rates of deposition and clearance determine the amount of drug present in the respiratory tract, thus the species selected for testing will influence the resulting dose to the lungs.

Different species breathing the same aerosol do not receive identical doses. As outlined above, there are variations in ventilation, collection efficiency, lung anatomy and clearance mechanisms among species that influence the local doses of therapeutic aerosols. Varying biological responses to the same aerosolized drug also reflect differences in the activation, degradation, excretion or mechanism of action of the compound in each species. The innate responsiveness of the analogous cell, tissue, or organ may also vary among species. An example is the case of the antituberculosis drug isoniazid, which is well tolerated at doses of 100 mg/kg in monkeys, whereas in dogs, doses of 20 mg/kg produce convulsion, respiratory failure and death. For example, it has been found that monkeys almost completely acetylate (thus inactivate) isoniazid, while dogs do not have that capability [82].

### **2.3 Methods of Aerosol Administration**

The efficacy of an inhaled drug is influenced by the amount of drug deposited at the target site. However, care must be taken when interpreting and comparing aerosol delivery efficiency data. For example, studies may report the efficiency of delivery in terms of the delivered dose, but this parameter does not consider the fraction of the dose deposited in the periphery of the lung that is required to exert action locally or the absorbed dose as in the case of drugs intended for systemic action. Figure 1 illustrates the relative magnitude of the drug amounts relating to the delivered, deposited and absorbed dose compared to the total amount of drug that is placed in the device for delivery as well as some examples of techniques to quantify each of these parameters.

The method of aerosol administration is a key factor in the design of animal studies for drug delivery to the lungs and will impact on the accuracy of the results obtained. Therapeutic compounds can be delivered to the lungs of animals by passive inhalation or direct administration methods that circumvent oropharyngeal deposition. Drug delivery of aerosols by passive inhalation in conscious animals is the most relevant method for extrapolating to therapy in humans. In inhalation toxicology there are methods to deliver accurate aerosol doses to conscious animals by passive inhalation [88], but their use to assess drug delivery may be impractical due to their complexity and the requirement of large reservoirs of drug, which are not relevant to small bolus doses. Direct administration methods are a good alternative, since a finite amount of material can be delivered to animals. However, since oropharyngeal and/or nasopharyngeal deposition are avoided in this approach the site of lung deposition is complicated by altered dependence on droplet or particle size effects compared with aerosol systems. Both methods of administration are discussed below.

### **2.3.1 Direct administration methods**

The main advantages of direct administration are that small and relatively large doses of drug can be delivered by these methods and that the dose delivered can be accurately measured. The main disadvantage is that the use of these methods for multiple or consecutive dosing may not be recommendable since they are performed under anaesthesia, and the insertion of the device may cause local irritation. Direct administration methods include liquid instillation, spray instillation and dry powder insufflation, but only the last two deliver drug in a form proxy to true aerosol. In general,

they involve visualization of the trachea of the animal with the help of a laryngoscope to place a thin stainless steel tube in the trachea, near the carina, to administer drugs. For small rodents, the procedure is straight-forward but for large animals, including rabbits, dogs, pigs and monkeys, some surgical procedures such as tracheotomy may need to be performed to accommodate the device.

Liquid instillation delivers drugs in the form of a liquid bolus. This method can cause significant stress in the subject, prevents uniform distribution throughout the lung and an unknown amount of the drug may be coughed up or swallowed. For spray instillation, the MicroSprayer™ (Penn Century, Philadelphia, PA) can be employed (Figure 2A). The atomizer in the tip of a long, thin, stainless steel tube generates a plume of liquid aerosol that can be deposited in the airways and deep lung. However, the droplet size is influenced by the physicochemical characteristics, such as viscosity of the solutions/suspension to be delivered. The Dry Powder Insufflator™ (Penn Century, Philadelphia, PA) has a small chamber that can be filled manually with a small amount of dry powder (Figure 2B). The powder is then dispersed by applying small "puffs" of air to the device using an empty plastic syringe and the amount of sample delivered to the lungs can be determined by gravimetric analysis. The insufflator has been used to deliver different powders from nanoparticles [89] to large porous particles [90,91]. The physicochemical characteristics of the powder, including particle size distribution, as measured by a variety of techniques have been reported to be essentially unaffected by passage through the device [67,90,92,89,93,94]. However, the position of the device in

the trachea of the animal during powder delivery may affect the percentage of powder deposited in the different lung lobes [95].

A range of therapeutic compounds that have been administered to the lungs with direct administration devices include: antibiotics[96,98], antiproteases [99,59,100], bronchodilators [101], chemotherapy [102,64], proteins and peptides [103,105], lung surfactant [106], vaccines [60,107] and gene therapy [108,68].

### **2.3.2. Passive inhalation**

Experiments designed to deliver aerosolized drugs to conscious animals by passive inhalation employ exposure chambers that can be for whole body, head-only and nose-only [88]. Each exposure type has its set of advantages and disadvantages. Advantages of the whole body inhalation systems include the ability to expose animals without restraint. The major disadvantage of these systems is the possibility of drug absorption by other routes including oral and percutaneous. A modified whole body exposure chamber was made using a conventional metabolism chamber with a polypropylene tube inserted to restrain the animal (Figure 3A) [109]. This chamber has the advantage of a small volume sufficient for the animal to receive a large dose of aerosol based on its inspiratory flow. Significant inter-individual variation might be anticipated as animals will vary in size, lung capacity and breathing parameters. Solution and suspension aerosols have been characterized and shown to retain the median particle size and distribution generated by the nebulizer after passing through the chamber [96,97]. Presumably this can be

attributed to the relatively small volume of the chamber and reduced tendency to lose particles by impaction and sedimentation [82].

The main advantage of head-only and nose-only exposure systems is the reduction or elimination of multiple exposure routes. Major disadvantages include adequate exposure seals for the face or neck of the animal and stress related to the restraint necessary for these studies [88]. Many types of nose-only exposure chambers are commercially available, including but not limited to those manufactured by CH Technologies (Westwood, NJ), Intox Products (Edgewood, NM), TSE GmbH (Badhamburg, Germany) and ADG Developments (Herts, UK). The exposure chamber manufactured by ADG (Figure 3B) is designed to deliver aerosols to approximately twenty rodents. Aerosols are continuously generated at a known concentration on a carrier air supply. Providing the mass flow (dose rate) and particle size and distribution remain constant each animal will be exposed to the same aerosol and the dose can be calculated from their respiratory flow parameters (tidal volume and breathing frequency). However, the inter-animal variation will play a role in dosing and it will still be necessary to independently evaluate the dose to the animal. This may involve conducting pharmacokinetic studies and inferring the dose or using *in-vitro* methods to estimate the dose at a given sampling flow rate. Aerosols of rifampicin solutions and suspensions of biodegradable particles have been delivered by this chamber for the treatment of tuberculosis [110,98].

Although contract research organizations conducting inhalation toxicology studies may use all forms of aerosol delivery devices (dry powder inhalers, pMDIs and nebulizers),

most published studies conducted with passive inhalation methods have employed nebulization to deliver drugs into the exposure chamber. A major limitation for the delivery of particles insoluble in biologically-friendly media by nebulization is the concentration that can be achieved in the suspension. Nebulization of highly concentrated suspensions may result in particle precipitation in the nebulizer and the delivery of very diluted aerosols. A new system for the delivery of powders to animals has been designed, the dry powder dispersion chamber [111]. The design is similar to that of the nose-only exposure chamber; however, the volume of the chamber is smaller and no air is introduced for the dispersion of powders in order to achieve a highly concentrated standing cloud of the therapeutic powder. This chamber has been used in pharmacokinetic and pharmacodynamic studies to deliver capreomycin for the treatment of tuberculosis in guinea pigs [111].

### **3. Methods to assess disposition and absorption of inhaled drugs**

Methods of aerosol characterization that can estimate the delivered dose *in vitro* have been widely described in the literature and are discussed later. Animal models employed to determine deposited dose and absorbed doses *in vivo* are discussed in this section. In general, these methods have been limited by the lack of sensitivity in the analytical procedures for the quantification of small concentrations of inhaled drugs in biological fluids and/or tissues.

### **3.1 Determination of local and systemic drug concentrations**

Once the drug has been delivered to the lungs, it is quantified by various methods depending on its desired effect.

In studies involving delivery of drugs for local action, determination of drug concentrations in the lung environment (epithelial lining fluid and tissue) together with plasma concentrations provide more valuable information. However, since the procedure is terminal for most animal models, large numbers of subjects are required making these studies costly and complicated by regulatory issues.

Bronchoalveolar lavage (BAL) is the sampling of the lower respiratory tracts by instillation of sterile saline and subsequent aspiration of the fluid [112]. Quantification of the drug in BAL can estimate concentrations in the epithelial lining fluid. The technique recovers cells, soluble proteins, lipids, and other chemical constituents from the epithelial surface of the lung. In human subjects BAL is performed under mild sedation. A flexible fiberoptic bronchoscope is employed to pass as far as possible in the right middle lobe or left upper lobe of the lung. Normal saline solution is introduced and aspirated. Then, the aspirated fluid is collected and analyzed. In large laboratory animals such as dogs and nonhuman primates, *in vivo* BAL is performed in a manner similar to that used in humans. BAL of small laboratory animals can be performed *in vivo* if required [113], but most lavage of rodents are performed on excised lungs. A catheter inserted into the trachea is used to instill the saline solution. Lavage volumes also vary for small laboratory animals, but usually the lavage volumes are approximately half of the total



lung capacity of the section of the lung lavaged. To evaluate drug concentration in BAL, two to four lavages might be performed to avoid excessive dilution of the drug. The concentration of urea in the BAL can be used as an endogenous marker [114] to estimate the sample dilution and accurately determine drug concentrations.

Quantification of the drug in lung tissue can also be performed, but may be challenging due to complicated extraction procedures that may compromise the integrity of the drug. Determination of drug concentrations in tissue may also provide information on drug metabolism in the lung, such as those reported for insulin [115].

For drugs intended for systemic action, concentrations are determined in serum or plasma. The blood collection sites are determined based on the volume and frequency of sampling required. In pharmacokinetic studies conducted in small rodents that require frequent blood sampling for a short period of time (usually less than a week), implantation of a cannula in the jugular vein is recommended. This requires a relatively complex surgical procedure. For studies that require less frequent sampling, localized bleeding in tail vein or artery, saphenous vein and the dorsal metatarsal vein can be used [116]. In rabbits, the central ear artery and marginal ear veins are commonly used [116]. In large animals, blood may be collected from a superficial implanted catheter from the femoral, cephalic or jugular veins [117,118]. Some studies have also been conducted in animals using jugular venipuncture [119,122]. However, the number of these studies may be limited by regulatory issues.

### **3.2 Pharmacokinetics models for the disposition of drugs in the lungs**

The pharmacokinetics of drugs after pulmonary delivery depends upon the dynamic interaction of different factors such as the site of deposition, clearance mechanisms, drug formulation (solid or liquid), dissolution rate, drug metabolism and mechanism of absorption [123]. In animals as in human subjects, the factors that will influence drug deposition are particle shape, size and distribution, hygroscopicity, static charge, anatomy of the airways and breathing patterns such as frequency and tidal volume. However, as reviewed in previous paragraphs, the two latter factors are different in animals, thus, different deposition patterns should be expected in the different animal species that may influence the pharmacokinetics of drugs. Clearance mechanisms include mucociliary clearance and endocytosis. Large interspecies differences have been reported in the lung clearance of inhaled particles [124,125]. However, lung models have been developed to extrapolate between different species [126]. Studies have also shown that the transport of particles by macrophages to the larynx that is substantial in rodents is rather small in humans [127]. Extensive reviews have been published on mechanisms of drug absorption in the lungs [128,129,6,130,9,10,131,12], thus they will not be included here.

Few models have been proposed to address lung residence time of soluble aerosols, due to the complexity of the processes involved and the factors that affect these. Byron [132] developed a mathematical method to determine residence times in the various regions of the respiratory tract as well as maximum deposition in these regions based on optimization of modes of inhalation and particle size. However, this model does not differentiate between drug released (pharmacologically active) and unreleased drug nor

does it separate absorption rate from dissolution rate. Therefore, Gonda [133] proposed a compartmental model that simulated the effects of release rate and multiple dosing on the duration of 'free drug levels' in the respiratory tract. This model also accounts for the possibility of accumulation of carrier materials during chronic administration as a function of drug release rate.

Pharmacokinetic (PK) parameters after aerosol administration of a drug can be calculated using compartmental or non-compartmental methods, and recently physiologically based PK models are being used more.

Compartmental methods consider the body as a system of compartments that usually are not physiologically or anatomically significant and determines the drug distribution between these compartments [134]. Compartments are arranged in a mammillary model and evaluated by intercompartmental transfer constants that assume instantaneous distribution. It is assumed that the rate of transfer between compartments and the rate of elimination of drug from a compartment follow first order or linear kinetics. The criteria to determine the best fitting model to a specific set of data are the Akaike criteria, the model selection criteria (MSC), the coefficient of variation (CV%), and the width of the confidence interval for each parameter estimate. The systemic disposition of most inhaled drugs is best characterized by one or two compartment models. The disposition of a few drugs has been characterized by a three compartment model with central elimination [135,123].

Noncompartmental methods consider a central homogeneous space, where drug input and elimination occur, and a heterogeneous space [135]. Drug is also sampled in the central space and the change of drug concentration over time in this space is usually regarded as a statistical distribution curve [136]. These methods are usually based on the estimation of the area under the drug concentration-time profile. Clearance, bioavailability, mean residence time and mean absorption time can be calculated by this method.

The main contribution of physiologically based pharmacokinetic methods is that they allow the prediction of drug concentrations in tissues in which samples cannot be easily collected. These models are well suited for making high-low dose, dose route and interspecies extrapolations by incorporating quantitative descriptions of the physiological and biochemical processes of the animal and the biochemical properties of the drug [123]. A classical PBPK consists of different compartments with each representing a particular organ. The organs of interest are selected based on different factors such as the route of administration of the drug, extent of body distribution, binding characteristics, elimination pathways, and pharmacodynamic activity.

### **3.3 Methods to calculate the rate of absorption and pulmonary bioavailability**

The rate of absorption has been determined by measuring the disappearance of drugs from the lungs or their appearance in systemic circulation. The rate of absorption is then calculated after curve fitting of semilogarithmic plots of the % remaining in the lung versus time or drug concentration over time [137]. The estimation of pulmonary rate of absorption is complicated by different situations such as gastrointestinal absorption of

drug swallowed following pulmonary administration, lack of sensitive methods to detect relatively small plasma and urine drug concentrations achieved under these circumstances and pulmonary metabolism.

The three methods most commonly used to estimate the rate and extent of absorption are the Wagner-Nelson, the Loo-Riegelman and the observational methods. The first two are based on curve fitting by compartmental analysis whereas the observational method is based on non-compartmental analysis [134,135]. The most important limitation of the Wagner Nelson method is that it is applied exclusively to drugs with one compartment disposition [138]. Loo-Riegelman proposed a modification in which the amounts of drugs absorbed at specific times could be calculated using two-compartment body models with first order elimination processes [139]. An inherent limitation of this method to determine pulmonary absorption is the intra-subject variability in inter-compartmental transfer constants between intravenous and pulmonary administration. The observational method is the most simple and practical method.  $C_{\max}$  and  $T_{\max}$  parameters, obtained directly from drug concentration versus time plots, are used to determine the rate of absorption.  $C_{\max}$  depends on both the rate and extent of absorption while  $T_{\max}$  takes discrete values and may be subject to reliability problems because its frequency is not normally distributed. Alternative measures include  $C_{\max}/AUC_{\infty}$ ,  $C_{\max}/T_{\max}$ ,  $C_{\max}/AUC_{\max}$  [135].

Caution should be exerted when interpreting the constant of absorption calculated for a drug delivered by the pulmonary route, since some characteristics of the drug, such as physical state (solid or liquid), solubility and possible interactions with excipients or

carriers can influence this parameter. For example, if the rate of drug absorption is limited by its poor solubility in the lung environment, the constant of absorption may be underestimated. Slow drug absorption may in turn influence the rate of elimination. This situation is known as flip-flop kinetics [136] and has been observed for tobramycin [140] and insulin [26]. Likewise, estimation of the rate of drug absorption from the lungs simply on the basis of  $T_{\max}$  is not recommended, since a short systemic half life can influence the magnitude of  $T_{\max}$ .

Differences in the rates of absorption have been observed among different species for hydrophilic drugs [141]. These compounds were absorbed 5 times faster in the mouse than in the rat, and 2.5 more slowly in rabbits than in rats. Conversely, lipophilic drugs were absorbed at the same rate in all species.

The term bioavailability describes the rate and extent of drug absorption. Methods for assessing bioavailability of drugs in the lungs are limited by analytical issues related to the low drug concentrations in lung tissue and plasma after aerosol administration. For some drugs such as  $\beta_2$ -agonists the issue of low plasma concentration can be circumvented by measuring drug in urine [142]. Oral charcoal has also been used to prevent GI absorption of drug that is swallowed after inhalation [143,144]. Complementary techniques such as imaging (discussed below) can be also used to estimate drug bioavailability. Pulmonary bioavailability can be calculated using AUCs after IV and pulmonary administration using the following equation.

$$F' = \frac{AUC_{0 \rightarrow \infty lung}}{AUC_{0 \rightarrow \infty IV}} * \frac{Dose_{IV}}{Dose_{lung}} * 100$$

It is important to note that the accurate pulmonary dose should be used in this calculation to avoid overestimation of bioavailability. Thus, direct methods of administration, in which the dose can be accurately measured, should be employed for this purpose. Interpretation of bioavailability values in animals should be made with caution, since depending on the species selected, this parameter could be overestimated or underestimated in humans. Pulmonary bioavailability of insulin is underestimated in rats and overestimated in rabbits and monkeys when compared to that of humans [145].

Although it is likely that factors such as differences in airway morphology and clearance mechanisms may have contributed to differences in insulin bioavailability in these species, the dosing techniques employed may have played a larger role. In the case of the rat studies [146] bioavailability may have been underestimated because intratracheal instillation was employed for dosing, most likely covering less than 5% of the lung surface, whereas in the rabbit and monkey studies [147,148] bioavailability may have been overestimated since a larger surface area is reached by nebulization.

When no analytical method can determine tissue or plasma drug concentrations, the magnitude of its effects (pharmacodynamics, PD) can be quantified to assess drug absorption. PD can be defined as the study of the biological effects and their mechanism of action. The objectives of the analysis of drug action are to delineate the chemical or physical interactions between a drug and the target cell and to characterize the full sequence and scope of actions of each drug [136]. Studies that evaluate the disposition of

insulin formulations for inhalation have employed drug pharmacodynamics as a measure of aerosol disposition [103,105]. These studies measured the decrease in glucose levels after administration of insulin formulations. The reduction of bacterial burden in affected organs after aerosol administration has also been used to assess the pulmonary disposition of anti-tubercular drugs [96,97,91,111].

### **3.4 Imaging of drug deposition and absorption**

Imaging is an exciting tool in drug development and its' role in the assessment of drug delivery is growing. With the improvements in imaging technology it is now possible to determine the delivery efficiency of a drug to its site of action and the resulting pharmacokinetics. Being able to characterise these parameters using non-invasive imaging of whole animals is particularly useful for assessment of inhalation delivery systems given the complex anatomy of the lungs and the complex biophysics governing aerosol deposition.

Drug distribution and kinetics of inhaled drugs in human lungs are primarily assessed by the imaging of radiotracers. Gamma scintigraphy is the most commonly used imaging method but single photon emission computed tomography (SPECT) and positron emission tomography (PET) are growing in popularity [149,150]. In general terms two major approaches have been adopted for imaging small animals: the first is the adaptation of clinical imaging technologies such as gamma scintigraphy, SPECT (Figure 4) [151] and PET to the smaller animal dimensions and the second is based on new evolving technologies based on whole-body photonic imaging[152].



A number of studies have used nuclear imaging to image radiolabelled drugs and particles delivered to small [153] and more commonly large [154,151,155,158] animals. Gamma scintigraphy has long been a mainstay of human lung studies and it could play a key role in the fundamental assessment of the effects of different devices/formulations on drug deposition and clearance in the lungs affecting drug absorption. There are difficulties and limitations to its use that include a need for careful validation of the tracer and potential problems in trying to quantify distribution with what is essentially a non-quantitative technique[150]. The capabilities of and technology behind human scanning have improved greatly over recent years, particularly with the emergence of SPECT and PET scans. SPECT is essentially planar imaging with a different gamma camera. In essence, planar images are acquired from several different angles and used to construct a 3-D distribution of radioactivity within the lungs (Figure 4) and clinically is becoming more popular than planar imaging, due to the improved data quality from both a qualitative and quantitative perspective. The disadvantages of this technique include the extra time to complete a scan and labelling of the drug of interest[150]. PET is a 3D functional imaging technique that provides accurate information on dose, distribution and kinetics of an inhaled radiotracer in the lungs[159]. This technique used to assess drug delivery to animals would provide a wealth of additional information including more accurate images detailing the regional distribution of the drug in the lungs, *in vivo* estimation of large airway/small airway deposition and functional imaging of physiologic and biologic processes *in vivo* [160]. The major advantage of PET is its high sensitivity without the penetration limitations of optical techniques. The most obvious limitations of this technique are the cost and complexity including the requirement for a local cyclotron

to generate probes, developing a labelling method for the drug in question, data analysis of complex studies and limits to quantification accuracy due to camera spatial resolution [150].

Another exciting new technology to study drug deposition and absorption through the lungs is real time *in vivo* fluorescence imaging. Temporal and spatial data can be collected from the same animal over multiple time-points allowing a real-time collection of data with significantly decreased animal numbers using drugs labelled with a fluorescent molecule such as a dye or quantum dot in whole-body living small animals [161]. It is a technique which is gaining increasing popularity amongst biological scientists but which could have enormous potential in drug and drug delivery system biodistribution studies going into the future [162]. Its comparative usefulness in inhalation PK studies has yet to be established.

#### **4. Inhalation Toxicology in Animals:**

With a growing number of candidate drugs (Table 1) and additives being assessed for drug delivery across the lungs (Table 2) animal models have a further fundamental role in inhalation toxicology. The main focus of inhalation toxicity studies is to determine local toxicities and evaluate the extent of systemic toxicity [163]. Inhaled substances can potentially have a range of deleterious effects from subtle changes in respiratory function, alterations in lung biochemistry and permeability to immunogenic effects and potentially mortality. The presence of enzymes in the lungs can also present the risk of toxic drug metabolites being formed after inhalation [164].

Choice of animal species is equally important for toxicological as pharmacological testing and the choice of animal models for inhalation toxicity testing is influenced by a number of factors including guideline requirements and practical considerations, such as exposure technology, expediency, previous experience [165] and differences in biological responses. Different species having different biological responses as exemplified by the varied reactions of monkeys and dogs to isoniazid outlined in section 2.2. and this impacts on the relevance of extrapolation of toxicity data to humans. Boyd and Neal showed that the LD<sub>50</sub> of Alpha-naphthylthiourea (ANTU) varied by more than two orders of magnitude between species when rodents, rabbits, dogs, cats, chickens and monkeys were compared [166]. These varying biological responses can be due to differences in deposition and clearance as well as differences in the activation, degradation, excretion, or mechanism of action of the substance in a given species[82]. There can also be animal specific factors that impact on the degree of exposure of the animal to the test substance. For example in rodents protective reflex reactions can limit exposure of the animal to the test substance being investigated [167].

Bolus doses of aerosol can be delivered to large animals such as dogs, pigs, sheep or primates from conventional inhalers but it is more likely that the early toxicological tests will be carried out in rodents. For rodents, spray instillation or powder insufflation is preferred to allow uniform dispersal of a defined dose in the lungs, to be followed by aerosol studies should the results prove positive. When conducting inhalation studies nose-only exposure is preferable to whole-body modes of exposure due to improved dose

control. Care must be taken in selection of the exposure system to minimize stress on the animals which can lead to artifacts [167]. Just as for PK/PD studies innate species differences such as: obligate nose breathing, differences in anatomical sizes, geometry and pulmonary function can lead to order of magnitude differences in upper respiratory deposition and consequently lung delivery. Accurate dose determination based on factors such as body weight, minute volume, percentage deposition, retention and absorption and pulmonary and extrapulmonary pathomechanisms[168] is essential as nominal dose can differ greatly from the actual dose delivered. In terms of extrapolation to humans the upper bound for the initial dose for a Phase 1 clinical trial is generally a fraction of the no-observed-adverse-effect level (NOAEL) in animals[163] but again the specific fraction is species dependent.

Indicators of toxicity can include changes in haematological, biochemical, neurological, physiological, immunological and exposure-related morphological effects, morbidity and mortality. The main focus of early stage acute preclinical studies designed to determine the feasibility of inhalation as a route of administration for an existing or new drug entity would be on local effects (as systemic effects would be unlikely after such short term exposure) and would include an assessment of pathological changes, inflammation and gross effects, which could potentially manifest themselves in the animal's behaviour [82]. Other markers of toxicity would include the wet and dry lung weights to evaluate fluid ingress into the lungs, histopathology to evaluate damage to lungs and cellular infiltration (monocytes, lymphocytes, polymorphonuclear cells). Even if such gross toxicity is not evident after only a single dose or small number of doses then it may be possible to

identify more subtle toxicological changes. Pulmonary function testing can identify changes in breathing parameters and analysis of the airway fluid using BAL (as described in section 3.1) can provide more subtle early markers of toxicity. BAL can be studied for changes in cell numbers and the presence of enzymes indicative of cell damage, e.g. lactate dehydrogenase, as well as the levels of inflammatory cytokines, e.g. interleukins, and reactive intermediates, e.g. nitric oxide. BAL can also be used to assess changes in airway permeability by determining the serum protein concentration in the lungs. Inhaled substances can affect the epithelial barrier by opening tight junctions or increasing cell membrane permeability. Immunological changes effected by inhaled substances can include local inflammation, bronchoconstriction, hypersensitivity and more damaging pathological changes such as basement membrane thickening all of which can impact on existing respiratory diseases and potentially underly progression to long-term disease states[82].

A full toxicological program involves acute inhalation toxicity testing, subchronic and chronic toxicity testing [82]. The main objective of acute inhalation toxicity is to determine the gross pathology and histopathology resulting from acute inhalation exposure to a drug but as described above monitoring of more subtle biochemical, physiological or immunological changes may prove useful at this stage of development[82]. In a sub-chronic exposure study the adverse effects resulting from repeated daily exposure of experimental animals to a chemical by inhalation for approximately 10% of their lifespan is determined. For mice this translates to exposure for 6hrs/day, 7 days a week for 18months, while for rats exposure is for 6hrs/day, 7 days

a week for 24 months. In a chronic exposure study the effects of a drug in a mammalian species following prolonged and repeated exposure are determined and effects that require a long latency period or are cumulative can be assessed. Other testing required can include hypersensitivity testing, carcinogenicity testing, reproductive toxicity studies and paediatric testing depending on the duration of treatment, target population etc. [163]. A detailed description of inhalation toxicity studies including respiratory physiology assessment, histopathology and bioassays is beyond the scope of this review but several comprehensive reviews are published [169,171,82,172].

There are a very limited number of excipients currently licensed for inhalation and a dearth of toxicological data which limits the range of formulation strategies that can be employed to enhance drug delivery and potentially absorption in a clinical setting. Improved toxicological assessment of such excipients could only benefit the field. A good example of such studies, were those conducted on HFA-134a as an alternative to chlorofluorocarbons for use in metered dose inhalers. Comprehensive preclinical inhalation toxicology study results indicated that HFA-134a was not considered toxic or oncogenic [173,174] and appeared to have no reproductive toxicity [175] and was therefore deemed a safe alternative. The potential of the lungs as a route of systemic administration of a drug will largely be determined by the toxicological implications of its inhalation and therefore good study design is critical.

## **5. Conclusion**

Comprehensive characterisation of drug delivery to the lungs is a complex task involving determination of delivered, deposited and for systemically targeted drugs absorbed dose. There is great scope for improved *in vitro* simulation of *in vivo* disposition and absorption of drug molecules after delivery to the lungs but in order to properly assess the pharmacokinetics effective use of animal models is still essential. This is particularly true for systemic delivery across the lung mucosa where the impact of normal lung perfusion, the complete epithelial barrier and clearance mechanisms are significant and can only be modeled in whole animals. With the range of drugs being assessed for systemic delivery via the lungs increasing and a vast array of new excipients and technologies being developed to assist drug absorption across the airway mucosa there is a growing need for assessment of toxicity and immunogenicity of inhaled drugs/ drug delivery systems. This is another important role of *in vivo* animal models. Using the appropriate toxicological techniques any structural, biochemical and immunological changes in the lungs after short and long-term drug administration must be assessed.

Great care must always be taken in rational animal study design but this is particularly true for pulmonary delivery. Because of the complexities and interspecies anatomical differences in the lungs it can be difficult to simulate the clinical situation of a human inhaling a drug. Appropriate choice of species and drug delivery method is therefore paramount. New devices designed specifically for inhalation animal studies and more sophisticated methods to determine drug deposition and absorption after inhalation are improving the information now derived from animal studies and hopefully reducing the

numbers of animals that will need to be used in the path to clinical development of drugs for systemic delivery via the lungs.

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**Tables:**

**Table 1: Examples of drugs being investigated for systemic delivery via inhalation**

<b>Drug candidate</b>	<b>Indication</b>	<b>Clinical status</b>	<b>References</b>
<b>Small molecules</b>			
Amikacin	Pneumonia	Phase II	[176]
Amphotericin B	Bronchopulmonary fungal infection	Phase III	[177]
Apomorphine	Erectile Dysfunction	Phase IIa	[178]
Epinephrine	Anaphylaxis	Phase I	[179]
Fentanyl	Pain	Phase IIb	[180]
Iloprost	Heart transplantation	Phase III	[181]
Nitroglycerine	Pulmonary hypertension	Phase II	[182]
Nitric Oxide adducts	Pulmonary hypertension	Phase II	[183]
Ribavirin	Viral infections	Approved*	[184]
Sumatriptan	Migraine	Preclinical	[185]
THC (Dronabinol)	Multiple Sclerosis, migraine, cancer, HIV	Phase II	[186]
Prochlorperazine	Migraine	Phase II	[187]
Treprostinil	Pulmonary hypertension	Phase II	[188]
<b>Proteins/Peptides</b>			
Insulin	Diabetes	Approved	[189]
Cetrorelix	LHRH antagonist	Preclinical	[190]
Cyclosporin	Immunosuppression	Phase 0	[191]
Follicle stimulating hormone	Fertility treatment	Preclinical	[192]
Parathyroid hormone	Osteoporosis	Preclinical	[193]
Heparin	Prevention of thrombosis	Preclinical	[194]
Calcitonin	Osteoporosis	Preclinical	[195]
Colony stimulating factorseg.Sargramostim	Metastatic cancer, Sarcoma	Phase II	[196]
Erythropoietin	Anaemia	Preclinical	[43]
Glucagon like peptide (GLP-1)	Diabetes	Phase I	[197]
Human recombinant F.IX	Hemophilia B	Preclinical	[198]
Immunoglobulin	Trigger/modulate immune response	Preclinical	[199]

Interferon-beta	Multiple sclerosis	Preclinical	[200]
LH-RH analogues	Endometriosis, prostate cancer	Preclinical	[201]

\* only for hospitalized infants with Respiratory Syncytial Virus infection

**Table 2: Strategies for enhancing systemic drug delivery via the lungs**

Strategy	Principle	Clinical Status	Reference
New generation inhaler devices	Improved delivery efficiency to site of absorption	Device dependant	[1517,36,18]
Molecular engineering: Modified peptides	Chemical modification of peptide of interest to protect it from lung peptidases.	Preclinical	[7]
PEGylation	Reduce macrophage recognition and subsequent proteolysis by conjugation of drug/protein with PEG.	Preclinical	[202]
Absorption promoting agents: e.g. Alkylglycosides, surfactants, bile salts, cyclodextrins, lipids and liposomes, amino acid analogues, carobopol, chitosan, protease inhibitors, dendrimers	Reduction in epithelial barrier to absorption	Phase II (liposomes) Preclinical (all others)	[42] [193,203209]
Bioadhesive polymers	Prolonged drug exposure to the mucosa	Preclinical	[51,207]
Bioengineering: Microparticles for inhalation composed of drug alone or with a range of excipients including PLGA, gelatin, albumin, PEG, sodium hyaluronate,  Diketopiperazine derivatives (Technosphere™),	Allow control over aerodynamic parameters and release of drug	Preclinical  Phase III	[49], [210,48,211213]



Preparation of large porous particles	Particle phagocytosis diminishes with increasing particle size	Phase 0 (Pulmosphere™) Phase III (AIR®)	[47]
Ligand targeting Drug conjugation to Fc domain of IgG	Conjugation of proteins to the constant Fc region of antibodies enhances absorption in the airways since airways are enriched with antibody transcytosis receptor mechanisms.	Phase I	[214,192,215]
Caveolae specific carriers	Conjugating the drug of interest to a protein/antibody which is transported in vivo via caveolae mediated transcytosis	Preclinical	[11]
Decreasing protein dose	Decreasing protein dose favors systemic absorption relative to local degradation	Preclinical	[23].

Table 3: Comparative Airway Structure: from replica casts (modified from [76])

Animal	Body mass	Airway branching	Trachea L/D(cm)	Tracheal bronchus	Major airway bifurcations	Airway L/D (ratio)	Typical number of branches to terminal bronchioles
Human	80kg	Relatively symmetric	12/2	Extremely rare	Sharp for about the first 10 generations;relatively blunt thereafter	2.2	14-17
Laboratory rat	0.3kg	Strongly monopodial	2.3/0.26	Rare	Very sharp and very high throughout the lung	1.5	12-20
Guinea pig	1kg	Monopodial	5.7/0.4	Absent	Very sharp and high	1.7	
Rabbit	4.5kg	Strongly monopodial	6/0.5	Absent	Sharp	1.9	
Beagle dog	10kg	Strongly monopodial	17/1.6	Absent	Blunt tracheal bifurcation	1.3	15-22
Rhesus monkey	2kg	Monopodial	3/0.3	Absent	Mixed blunt and sharp	2.6	

Table 4: Morphometric parameters in the alveolar region of normal mammalian lungs  
(modified from [76])

Fischer 344 Rat		Dog	Baboon	Human
Body weight, kg	$0.29 \pm 0.01$	$16 \pm 3$	$29 \pm 3$	$79 \pm 4$
Lung volume, ml	$8.6 \pm 0.31$	$1322 \pm 64$	$2393 \pm 100$	$4341 \pm 284$
Total number of cells/lung, $10^9$	$0.67 \pm 0.02$	$114 \pm 13$	$99 \pm 9$	$230 \pm 25$
<b>Total lung cells, %</b>				
Alveolar Type I	$8.1 \pm 0.3$	$12.5 \pm 1.7$	$11.8 \pm 0.6$	$8.3 \pm 0.6$
Alveolar type II	$12.1 \pm 0.7$	$11.8 \pm 0.06$	$7.7 \pm 1.0$	$15.9 \pm 0.8$
Endothelial	$51.1 \pm 1.7$	$45.7 \pm 0.8$	$36.3 \pm 2.4$	$30.2 \pm 2.4$
Interstitial	$24.4 \pm 0.7$	$26.6 \pm 0.7$	$41.8 \pm 2.7$	$36.1 \pm 1.0$
Macrophage	$4.3 \pm 1.0$	$3.4 \pm 0.6$	$2.3 \pm 0.7$	$9.4 \pm 2.2$
<b>Average cell surface area, <math>\mu\text{m}^2</math></b>				
Alveolar Type I	$7287 \pm 755$	$3794 \pm 487$	$4004 \pm 383$	$5098 \pm 659$
Alveolar type II	$185 \pm 56$	$107 \pm 15$	$285 \pm 85$	$183 \pm 14$
Endothelial	$1121 \pm 95$	$1137 \pm 127$	$1040 \pm 209$	$1353 \pm 67$

Table 5: Tidal volumes and breathing frequencies among laboratory animals (modified from [216]).

Species	Respiratory rate (breaths/min)	Tidal volume (ml)	Minute volume (liters)
Mouse	163	0.15	0.023
Rat	85	1.5	0.10
Guinea Pig	90	1.8	0.16
Rabbit	46	21.0	1.07
Dog	18	320.0	5.2
Monkey (rhesus)	40	21.0	0.86

**Figure Legends:**

Figure 1: Relative magnitude of the drug mass considered in the parameters used to assess the efficiency of aerosol delivery and examples of techniques to quantify them.

Figure 2: Figure 2: (A) The MicroSprayer™ and (B) Dry Powder Insufflator™  
(Reproduced with permission of Penn-Century, Inc., Philadelphia, PA)

Figure 3: Examples of (A) The Modified Whole Body exposure chamber (modified from [109]) and (B) The noseonly exposure chamber (ADG Developments, Ltd, Herts, UK)

Figure 4: SPECT scan 3 h after inhalation. *A*: series of transversal (apex-to-base) and coronal (dorsal-to-ventral) slices of  $^{99m}\text{Tc}$ -PSL retention in lungs of *dog 4* around level of carina. Slices were calculated at a distance of 2 mm apart. Retention of  $^{99m}\text{Tc}$ -PSL is overlaid in color scale over a ventilation scan in gray scale performed earlier. *B*: series of sagittal (right-to-left lateral) slices of  $^{99m}\text{Tc}$ -PSL retention in lungs of *dog 4* and planar image from dorsal view of  $^{99m}\text{Tc}$ -PSL deposit in lungs and PSL accumulation in trachea at site of ciliar obstruction because of inflation of cuff of endotracheal tube. Planar image was taken at beginning of SPECT scan 2.5 h after end of inhalation (from [151]). (PSL=polystyrene particles).

**Figures:**  
Figure 1:

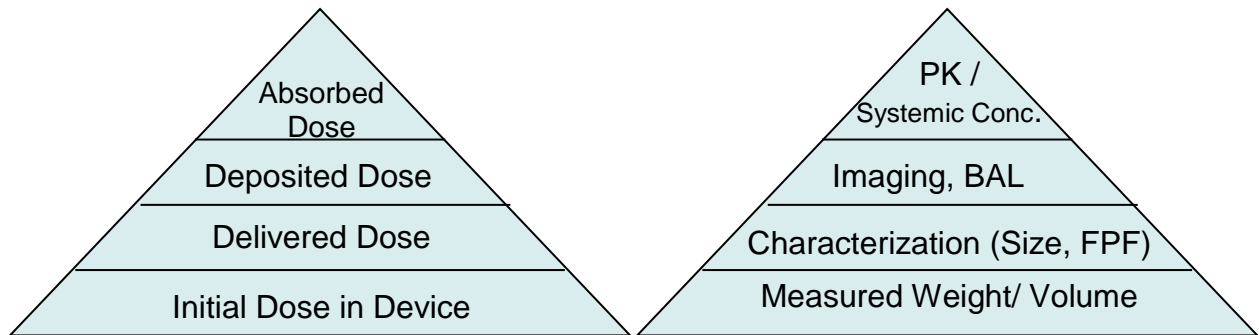


Figure 2:

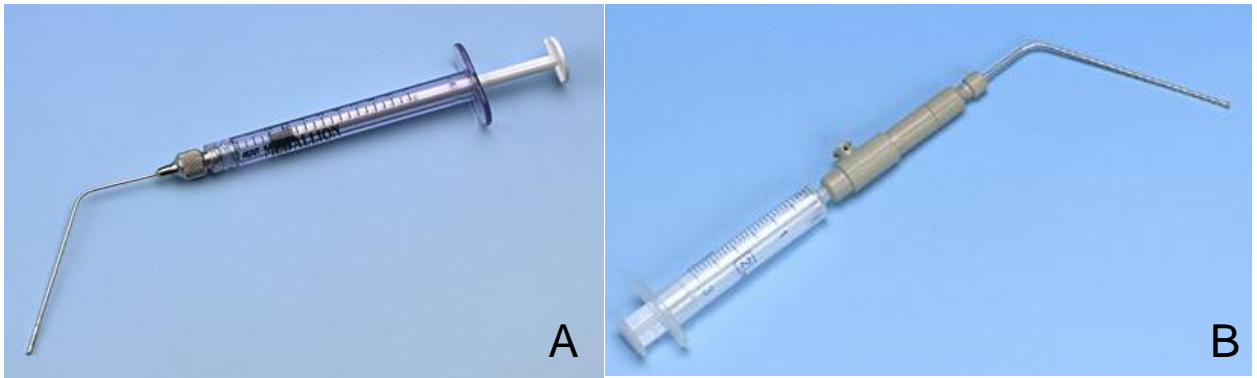


Figure 3:

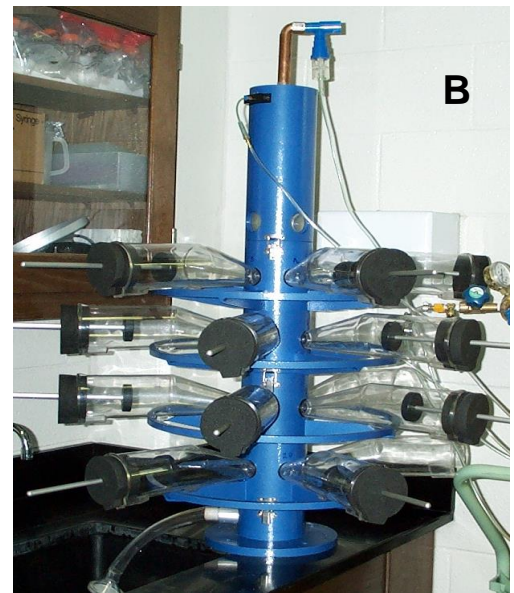
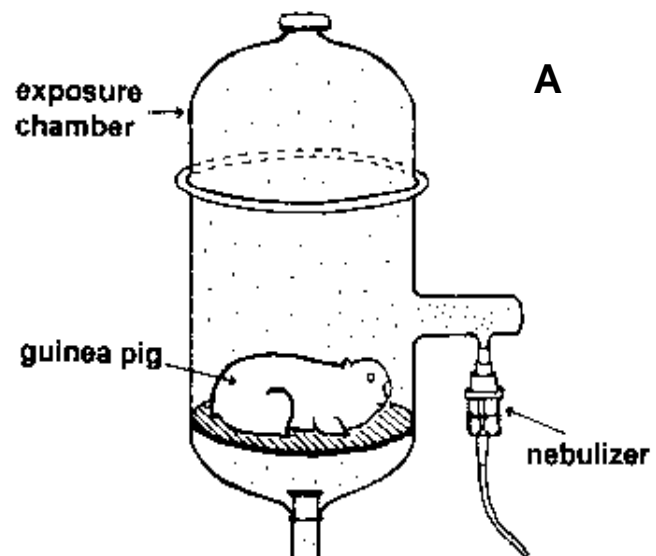
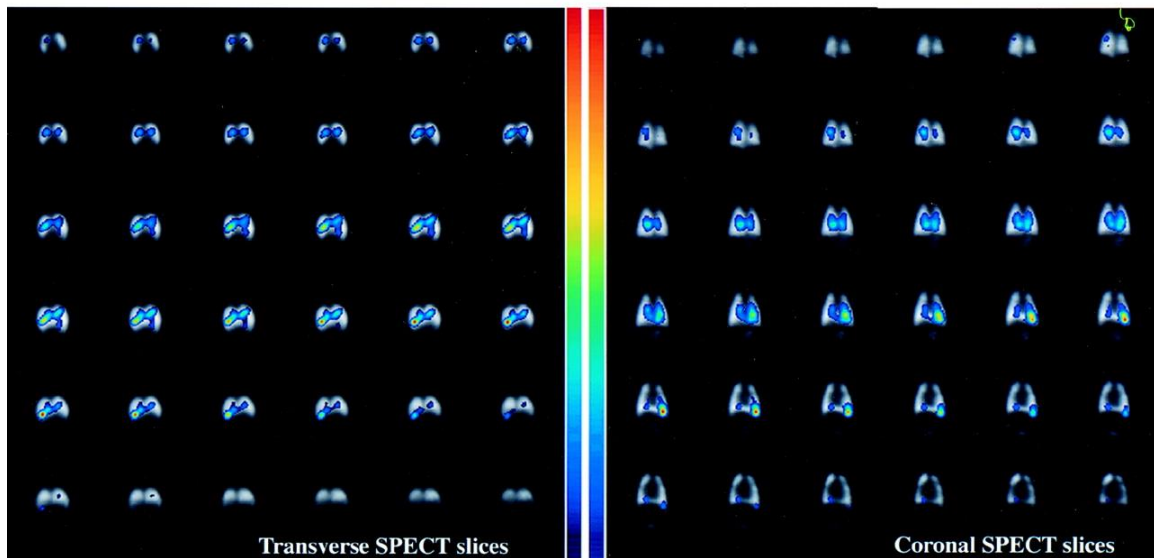




Figure 4:  
A



B

