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In vitro and *in vivo* testing methods for respiratory drug delivery

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Importance of the field: Successful respiratory drug delivery for local and systemic purposes is predicated on the availability of *in vitro* and *in vivo* methods for determining drug delivery and disposition following respiratory administration.

Areas covered in this review: In this review, the relevance of new *in vitro* and *in vivo* methods for screening respiratory drug delivery is discussed. Specific topics covered include *in vitro* particle size characterization, *in vitro* dissolution test methods for respiratory formulations and *in vitro* respiratory absorption and disposition screening methods. Furthermore, *in vivo* respiratory dosing methods, *in vivo* respiratory aerosol deposition and drug absorption screening methods, and correlation between *in vitro* and *in vivo* methods are reviewed.

What the reader will gain: After reading this article, the reader will have an enriched knowledge regarding the various *in vitro* and *in vivo* testing methods for respiratory drug delivery. Most importantly, this paper will make it possible for readers to appreciate the strengths and weaknesses of each test method, which in turn will assist them in selecting specific methods that suit their scientific needs.

Take home message: New *in vitro* and *in vivo* methods for screening respiratory drug delivery are indispensible, especially from the respiratory drug development and quality control perspective. Each method has unique advantages and disadvantages that influence method selection and data interpretation. Although *in vitro* methods are used during drug development, they augment rather than substitute *in vivo* methods.

Keywords: cell culture, drug testing methods, *in vitro*, *in vivo*, inhalation aerosol, respiratory drug delivery

*Expert Opin. Drug Deliv. (2011) **8**(1):57-69*

1. Introduction

The lung has traditionally been targeted for the treatment of various respiratory diseases, including asthma, emphysema, chronic obstructive pulmonary disease (COPD), cystic fibrosis and other respiratory pathological abnormalities [1,2]. Over the past few decades, the attractiveness of the lung as a target for systemic drug delivery has increased, especially for therapeutic proteins and peptides [3,4]. This attention is because of the numerous advantages of pulmonary drug delivery, which include large alveolar surface area suitable for drug absorption, low thickness epithelial barrier, extensive vascularization, and the relatively low enzymatic metabolic activity in addition to the absence of the first-pass effect [5]. Drug absorption in the lung may be affected by aerosol particle size distribution and deposition within specific regions of the respiratory tract [6].

10.1517/17425247.2011.543896 ISSN 1742-5247

Article highlights.

- Particle size and size distribution of inhaled drugs affect drug deposition and therapeutic outcome. *In vitro* respiratory particle size determination methods that include impaction and light scattering principles play a major role in respiratory aerosol particle size characterization.
- The aerosol particles of the inhaled drugs in the non-ciliated areas of the lung dissolve in thin fluids, and the dissolved drugs become available for absorption. New *in vitro* dissolution methods have been developed for investigating respiratory drug dissolution within a thin layer of fluid *in vitro*.
- Several tissue culture methods (A549, Calu-3, 16HBE14o-, BEAS-2B, primary alveolar and tracheal cultures) have been developed for screening respiratory drug delivery. Each tissue culture method can be used to investigate drug absorption, transport, metabolism and toxicity. Some of the cell culture methods have been adapted with particle deposition and drug dissolution components to reflect *in vivo* drug deposition, dissolution and absorption.
- Based on several factors that affect drug deposition in the lung, several methods have been used for dosing experimental animals during *in vivo* studies. These methods affect drug absorption, disposition, data interpretation and extrapolation.
- Several methods including gamma scintigraphy, single-photon-emission computed tomography, positron-emission tomography and aerosol bolus methods have been used to determine regional and total particle deposition in the lung. Pharmacokinetic and pharmacodynamic methods are the most useful methods for measuring respiratory drug absorption and effectiveness.
- Although *in vivo* drug deposition is a very complicated process, research has shown that *in vivo* drug deposition and absorption can be investigated using both *in vitro* and *in vivo* methods. With proper validation and careful experimentation, these new methods discussed in this review are very useful not only for preclinical drug screening, but also for quality control and assurance during respiratory drug manufacturing processes.

This box summarizes key points contained in the article.

The respiratory tract is organized into upper and lower airways. The lower airways diverge into several branches or bifurcations that affect particle impaction and deposition. The larger the particle size, the greater the velocity of incoming air, the greater the bend angle of bifurcations and the smaller the airway radius, the greater the probability of deposition by impaction [7,8]. Furthermore, the pulmonary delivery of some formulations (e.g., dry powders) is difficult because they tend to agglomerate into larger aggregates owing to van der Waals and electrostatic forces between particles, thus decreasing the airflow properties of the powders and their subsequent deposition in the deep lung [9]. Particle clearance mechanisms including mucociliary clearance, phagocytosis and enzymatic degradation decrease the extent of drug absorption from the lung [10]. Aerosol characteristics such as particle diameter, particle density, hygroscopicity and electrical charge and patient-related factors such as age, stage of pulmonary disease, breathing pattern and morphometry of the lower airway influence aerosol deposition and therapeutic effectiveness of inhaled medications [3,11]. Other factors such as velocity of aerosol entry, quality of aerosol deposition, binding characteristics of the aerosol on the alveolar surface, and the quality of alveolar capillary bed and its subsequent vascular tree also affect considerably respiratory drug delivery [12].

Considering the complexity of the lung in terms of its anatomical organization, chemical/enzymatic barriers, geometry and particle clearance mechanisms, optimization of drug delivery using *in vitro* and *in vivo* methods is very challenging. Nevertheless, new *in vivo* and *in vitro* methods have been developed for investigating respiratory drug delivery. Many of these methods pay particular attention to the particle size and distribution of the inhaled drug aerosol, as well as delivery devices and methods for accurately estimating drug absorption and disposition. Several *in vitro* and *in vivo* testing methods for respiratory drug delivery, especially methods for aerosol generation, particle size analysis, and methods for determining drug absorption and disposition are very useful in respiratory drug development. In this review, the relevance of new *in vitro* and *in vivo* methods for screening respiratory drug delivery is discussed. Specific topics covered include *in vitro* particle size characterization, *in vitro* dissolution test methods for respiratory formulations and *in vitro* respiratory absorption and disposition screening methods. Furthermore, *in vivo* respiratory dosing methods, *in vivo* respiratory aerosol deposition and drug absorption screening methods, and correlation between *in vitro* and *in vivo* methods are reviewed.

2. *In vitro* particle size characterization methods for respiratory drugs

Clinically, respiratory drug delivery is achieved by means of inhalation of aerosols. Particle size and size distribution of the aerosols play an extremely important role in airway drug deposition and therapeutic outcome [13]. The ideal size for a therapeutic aerosol is not known precisely, but it may be assumed that the mass median aerodynamic diameter (MMAD) should not be > 5 µm to penetrate the tracheobronchial tree and smaller airways [14]. The rapid assessment of aerosols produced by medicinal inhalers is highly desirable from several standpoints, including the assurance of product quality, the development of new delivery systems, and the need to meet an increasing requirement by regulatory bodies for reliable *in vitro* performance data [15]. During respiratory drug development and testing, inhalational aerosols are tested using various methods, including aerodynamic measurements based on inertial separation and optical methods using microscopy or light scattering principles [16].

2.1 *In vitro* particle size measurement using inertial impaction methods

Cascade impactors (CIs), including the multistage liquid impinger, are by far the most widely used methods for *in vitro* determination of particle size distribution of respiratory aerosols in medical inhalers during product development, batch release and in applications with add-on devices [17]. This approach is the gold standard approved by regulatory agencies for particle size analysis during respiratory drug delivery formulation and quality control [17]. The preference of this method stems from the fact that direct measurement of aerodynamic particle size is possible with it. Furthermore, with this method, the mass of the drug in the formulation within different size ranges can be determined without interference from other excipients in the formulation. Aerodynamic particle measurement is of practical use because of its relationship with the mass of aerosol particles deposited in the respiratory tract [18]. Cascade impactors measure the mass distribution of the inhaler-produced aerosol in terms of aerodynamic diameter, a size parameter that takes in to account the effects of both particle density and shape on particle motion [19].

Stein [20] conducted some experiments to illustrate the challenges associated with measuring dynamic aerosol particles. It was demonstrated that the size distribution of metered-dose inhaler aerosols changes substantially during the measurement process. The measured size distribution was shown to be dependent on the degree of evaporation that has occurred before size measurement. Also, the degree of evaporation before the measurement also influences the number of modes present in the measured size distribution. The dynamic nature of the process implies that robust statistical methods are necessary for estimating particle size distribution when using this approach. According to Dunbar and Mitchell [19], a CI-generated mass distribution can be directly related to aerodynamic diameter by expressing the mass collected by each size-fractionating stage in terms of either mass frequency or cumulative mass fraction less than the aerodynamic size appropriate to each stage. Hence, the analysis of the aerodynamic diameter as a continuous variable allows comparison of mass distributions obtained from different products and CI designs, as well as providing input to *in vivo* particle deposition models. Although statistical methods play an important role in accurately estimating the size distribution of mobile particles, the mixing of droplets and entrained air streams, heat and mass transfers that occur downstream in *CIs* actually determine the final aerosol size distribution measured by the cascade impactor. The influence of these variables on size measurements may be reduced significantly by refrigerating the impactor down to 5°C before measurement [21].

In an attempt to improve the data quality based on CI studies and the efficiency of the method, Bonam *et al.* [22] catalogued in a systematic way the available information about factors that may influence the outcome and variability of CI measurements of pharmaceutical aerosols for inhalation, such as those obtained from metered-dose inhalers (MDIs), dry powder inhalers (DPIs) or products for nebulization. Based on their study, the authors concluded that the awareness, understanding and consideration of all the factors should aid in the development of optimized CI methods and appropriate quality control measures for aerodynamic particle size distribution of pharmaceutical aerosol measurement that is in line with the current regulatory initiatives involving quality-by-design [22].

Considering the labor-intensive nature and time consumption aspect of CIs, several adaptations have been done to the *CIs* to increase their efficiency. Such modifications include automation, addition of induction port, pre-separators, breathing simulators and attachment of inhalers for nasal delivery. Other attempts that have been made to improve the quality of data from *CIs* include the introduction of a next-generation pharmaceutical impactor (NGI) and the use of abbreviated impactor measurement. The NGI was developed to serve the needs of the pharmaceutical industry better than the more commonly used Anderson cascade impactor (ACI), which was originally developed to measure size distributions of viable airborne particles [23,24]. To demonstrate the edge that the NGI has over the ACI, Guo Changning *et al.* [25] compared particle size distribution and dose delivery characteristics for the Combivent® using the ACI and the NGI by investigating the influence of flow rates, induction ports and operators. Their results show that, at their normal operation flow rates, the ACI (28.3 l/min) and the NGI (30 l/min) yield a similar particle size distribution and dose delivery profile for the MDI product tested, which implies that they deliver equivalent analysis results. However, this similarity was lost at a higher flow rate of 60 l/min owing to particle bounce. Increasing the flow rate enhances this effect and results in smaller MMAD values. Compared with the smooth surface of the ACI plates, the rough NGI collection cup bottoms are less sensitive to particle bounce and re-entrainment. For this reason, collection performance degrades less for the NGI than for the ACI at higher flow rates. The results of their studies imply that special care must be taken to eliminate any contingency in experimental parameters and selection of ancillary devices such as the pre-separator, induction port or throat to ensure good repeatability and reproducibility of inhalation drug testing.

The abbreviated impactor measurement (AIM) concept has been promoted as a solution to the labor-intensive full-resolution CI methodology for inhaler aerosol particle aerodynamic size measurement [26]. A study by Mitchell *et al.* showed that both abbreviated impactors were found to be substantially equivalent to the full-resolution ACI in terms of extra-fine and fine particle and coarse mass fractions used as metrics to characterize the particle size distribution of ethanol containing MDI-produced aerosols when sampled at 28.3 l/min, provided that precautions were taken to coat collection plates to minimize bounce and entrainment. Nevertheless, the authors cautioned that before implementing this type of simplified methodology for routine use in inhaler

product characterization, the system suitability must be evaluated on a product-by-product basis to establish substantial equivalency [27].

Although the CI is used for estimating aerosol particle size, Dunbar and Mitchell [19] emphasized some important facts concerning this method. For example, there are differences between particle deposition in the lung and CIs. Whereas the lung separate particles by means of combined processes of inertial impaction, sedimentation and diffusion within an envelope of continuously varying flow rate, *CIs* separate particles mainly by inertial impaction at a constant volumetric flow rate that enables the measurement of aerodynamic particle size distribution. These differences imply that the CI cannot be used directly as an *in vitro* surrogate to mimic deposition in the respiratory tract. Rather, CI should be used to measure mass-weighted aerodynamic diameter distribution, which can be used to estimate *in vivo* deposition using appropriate mathematical models [19].

2.2 Particle size measurement using optical methods

Alternative methods to the CI that may be used for particle size analysis for respiratory drug delivery include laser diffraction, light scattering, laser Doppler and time-of-flight methods. This section focuses more on the laser diffraction method as it is the only alternative method to *CIs* that have been used extensively for respiratory aerosol particle size characterization. The amount of light scattered by a particle is a function of its size and forms the basis for classifying particles in these systems. The use of lasers rather than white light provides better resolution and increased accuracy, both for the diffraction systems, which characterize a slice of the MDI aerosol spray, as well as the single particle counters, which utilize the signals produced by the particles to measure their velocity as they cross the laser beams [15]. Laser diffractometers (LDs), alternatively and more correctly termed low angle laser light scattering (LALLS) instruments, are a class of particle size analyzer that operates on the basis of the interpretation of the light scattering pattern that is set up by an ensemble of either solid particles or liquid droplets in a collimated beam of coherent light [28]. Unlike the *CIs* where particle sizes are measured directly, the LD measures volume equivalent diameter. Volume- and mass-weighted size distributions are equivalent, on the basis that droplet density is constant irrespective of size of the liquid droplets that are produced from nebulizers [29]. On this basis, it is possible to compare CI data directly with LD data without data transformation from a number-weighted basis that is necessary in other optical single-particle detection methods such as time-of-flight or phase-Doppler particle size analysis [15]. Although particle size analysis has traditionally been undertaken by cascade impactors on account of the direct assessment of active pharmaceutical ingredient(s) (APIs) that is possible by this method, laser diffractometry is less labor-intensive, more rapid, and can be a less invasive procedure [30]. As suggested by Mitchell and Nagel [30], the technique provides meaningful results, as long as precautions are taken to validate the method. Recently, this method was used to characterize the potential use of new propellants (Perfluorobutylpentane [F4H5], Perfluorohexylhexane [F6H6], Perfluorohexyloctane (F6H8) and Perfluorohexyldodecane [F6H12]) for formulating MDIs [31]. Furthermore, the method was used to characterize dry powder aerosols generated by standardized entrainment tubes from drug blends with lactose monohydrate, particle aerosolization and break-up in dry powder inhalers (I-neb adaptive aerosol delivery system) and the interaction between the oropharyngeal geometry and aerosols via pressurized metered-dose inhalers [32-34]. Despite the usefulness of the technique, it has some limitations. The major limitation with respect to the application of the method for nebulizer droplet characterization is the lack of traceability to the mass of APIs, as chemical assay is not performed as part of the measurement process [15]. Considering this disadvantage, this method is not suitable for formulations whose API is contained in particles suspended in liquid medium or solutions without validation [35,36]. Also, care is required to establish valid operating conditions, particularly when using laser diffractometry either for the first time, or with a new formulation/inhaler [15].

Recently, the white light aerosol spectrometer has been proposed as an alternative method to multistage cascade impaction for the analysis of particle size distribution (PSD) that allows measurements in high concentration liquid solutions by single particle measurement [37,38]. For aerosols from solution-based pressurized metered-dose inhalers, both the apparent density and the dynamic shape factor of the dry solid particles come into account for correlation of aerodynamic particle size to scattered light equivalent diameter [39].

3. *In vitro* dissolution test methods for inhalation formulations

Dissolution testing allows the investigation of the ability of a solid substance to enter a solvent based on the affinity between the solid substance and solvent, which provides some predictive estimates of the solid substance's absorption behavior *in vivo* [40]. The aerosol particles of the inhaled drug that enters the pulmonary region in the non-ciliated area undergo dissolution in the lung fluids, and the dissolved fraction of the dose becomes available for absorption across the alveolar membrane [41]. A range of pharmacopoeia methods exist for the testing of conventional solid dosage formulations. However, these methods were designed to mimic the gastrointestinal tract and as such are based on 'sink' conditions and may not be appropriate for dissolution testing for respiratory drugs [42]. Designing a standardized method applicable to the lung is an arduous task, as the lung has unique features that are difficult to replicate *in vitro*, such as the extremely small amount of aqueous fluid and lung surfactant, as well as the extra requirement to collect the API fraction from the entire formulations before dissolution tests [43]. At present, there are no generally

accepted dissolution methods for inhaled drugs. Nevertheless, several authors have used the United States Pharmacopoeia (USP) methods either without modification [44] or with some adaptations for respiratory drugs screening [45].

McConville [43] developed a new standardized test method to characterize the dissolution properties of formulations intended for pulmonary delivery using a modified commercially available dissolution tester by incorporating a membrane-containing cassette designed to enclose previously air-classified formulations, so that they could be uniformly tested in the dissolution apparatus. This study demonstrated that their method was reproducible and may be used to evaluate the dissolution properties of pharmaceutical inhalation products following their aerodynamic particle classification. In another study, Salama *et al.* [42] evaluated three methods for the measurement of drug release from inhalation-controlled release formulations. The methods studied include: i) the conventional USP Apparatus 2 paddle method [46]; ii) the modified USP apparatus 4 flow-through system [47]; and iii) a diffusion model based on a transdermal diffusion Franz cell [48]. The analysis of the release profiles suggested that the diffusion data fitted a Higuchi diffusion model. Based on the physiology of the lung, the authors suggested that this model and method was the most useful for investigating respiratory formulations. Although data from the study suggested that the modified Franz cell apparatus was the most promising method, further studies are needed to correlate this *in vitro* dissolution test with *in vivo* data. In addition to these methods, other variations based on a shaking incubator [49,50], twin-stage impinger [51] and dissolution cell [52,53] have been reported.

Recently, Arora *et al.* [54] suggested that an ideal dissolution method should enable the kinetic assessment of dissolution for defined-size and respirable aerosol drug particles generated from inhaler products, into the limited volume of the stationary fluid, like actual aerosol particle dissolution on the lung surface [55]. The authors developed a unique dissolution testing method for aerosol drug particles generated from commercial inhaler products, with a focus on collection of defined-size and respirable particles and their dissolution in the limited volume of the stationary aqueous fluid. Following Davies and Feddah [56], they used the compendial ACI to collect aerosol drug particles in the defined aerodynamic diameters of 2.1 – 3.3 and 4.7 – 5.8 µm by impaction on the filter membranes placed on stages 4 and 2, respectively [55]. A scheme of the innovative method, including the dissolution insert, is shown in **Figure 1**. Through this unique dissolution system, the inhaled corticosteroid aerosol particle dissolution in the limited volume of aqueous fluid was shown to differ kinetically owing not only to the solubility, but also to aerosol mass, size and formulation.

4. *In vitro* respiratory absorption and disposition screening methods

Primary cell culture of lung epithelium has been developed from most species, including rats and humans for drug delivery studies. Also, several human lung epithelial cell lines derived from cancers or transformed by viruses, such as A549, Calu-3, 16HBE14o- and BEAS-2B, have been tested recently for their validity and usefulness; by virtue of their continuous nature in culture, these cell line systems may offer more reproducibility and ease of use for investigators [57]. Specifically, each of these systems is capable of measuring absorptive drug transport and discriminating between passive diffusional flux and active transport mechanisms [58]. They are also useful for toxicity and metabolism studies [59,60]. Several review articles have been published on the relevance of these *in vitro* tissue models for respiratory drug delivery [61-64]. Already published reviews on respiratory cell culture models have covered most aspects of tissue culture-related applications in drug delivery. An aspect of tissue culture models for respiratory drug delivery that has not been reviewed extensively is the methods used for exposing drug formulations to the cells. Considering the complexity of respiratory drug delivery *in vivo*, especially the roles of particle size, dosimetry, presence of low liquid film on respiratory epithelium compared with the large volume of the gastrointestinal tract, and the non-dynamic nature of the process (no peristalsis), it is important to examine critically new methods for aerosol delivery to cultured respiratory epithelial cells.

In 2003, Fiegel *et al.* [65] designed an experiment to investigate the impingement of large porous particles on lung epithelial cells *in vitro*. According to the authors, the ability to optimize new formulations for pulmonary drug delivery has been limited by a lack of relevant *in vitro* models that take particle deposition in various regions of the respiratory tract into consideration. Conventional 'complete immersion' methods used to characterize particle water uptake rates, polymer degradation kinetics, drug diffusion rates and particle dissolution rates may not be relevant for particles designed for inhalation because of the extremely thin aqueous layers found in the lungs [66]. In their study, one tissue culture well was placed directly under the second-stage nozzle of an Astra-type liquid impinger that was sealed. Microparticles were aerosolized onto the monolayers from number 2 gelatin capsules using a Spinhaler device. This approach was similar to the method described by Forbes *et al.* for nasal delivery [67]. Another study by Cooney *et al.* [68] described the blending of the dynamics of aerosol delivery and *in vitro* simulated lung model to evaluate the transport properties of a series of molecular mass marker compounds across human-derived bronchiolar epithelial cell monolayers. In the study, the authors used an Andersen cascade impactor as a delivery platform for depositing size-segregated particles to monolayers of small airway epithelial cells and Calu-3 cells. In a recently related study, Bur *et al.* noted that submersed cell culture systems are of limited value for screening the interaction between inhaled particles and cells lining the alveolar space because significant differences arise from the application of particles in solid-state on a more or less dry cellular surface instead of the application of a suspension or even solution of particles

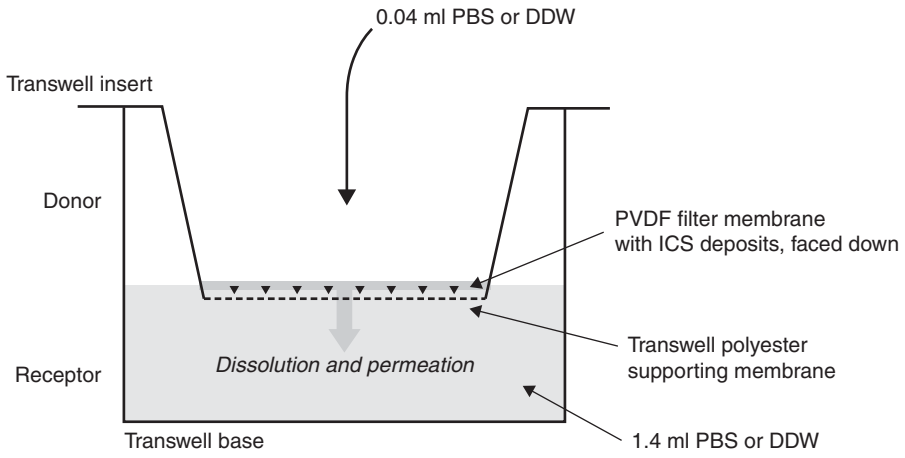

Figure 1. Dissolution and permeation for the defined-size aerosol particles of inhaled corticosteroids (ICS) in the Transwell® system. The PVDF filter membrane with the deposited ICS was placed, with the deposited drug face down, onto the donor compartment. Then, 0.04 ml of aqueous fluid was added over the filter membrane to initiate drug dissolution and permeation into the 1.4 ml receptor fluid, while the entire system was maintained at 37°C and near 100% relative humidity inside the incubator.
Reproduced with permission from [56].

on submersed cells [69]. In their studies, the authors used a modified Astra-type multistage liquid impinger (MSLI) with integrated bronchial cell monolayers to study deposition and subsequent drug absorption from *in vitro* models of the human airway epithelial barrier. The integration of the impinger with cultured cells was achieved by using inverted cell culture of Calu-3 cells, which was cultivated upside down. This integration had no effect on barrier function, morphology and viability of the cells. Although this was an upgrade compared with the studies described by Fiegel *et al.* and Cooney *et al.* [65,68] because of increased particle deposition efficiency and reduced cell stress owing to reduced turbulence, the inverted approach led to lower electrical resistance.

Also recognizing the importance of particle deposition and respiratory drug absorption, Grainger *et al.* conducted a comprehensive investigation to determine the transepithelial transport rates of compounds after deposition as aerosolized particles onto respiratory cell layers [57]. The authors used the twin-stage impinger (TSI) to deposit potentially respirable particles (aerodynamically < 6.4 µm) of varying molecular mass dextrans labeled with fluorescein isothiocyanate (FITC-dex) onto Calu-3 cells, a model of the bronchial epithelium. Based on the results of their studies, the authors concluded that the use of the TSI and the Calu-3 cell line for the assessment of compound dissolution and transport rates after particle deposition may allow more realistic analyses to be made with respect to the *in vivo* situation. This approach was taken further by Bur *et al.* [70]. They investigated the effects of particle size, apical liquid volume and deposition technique on drug transport across respiratory epithelial cells. The results of their studies showed that epithelial transport rates vary *in vitro*, depending on the amount of apical liquid and the particle deposition technique. Their studies also showed that the dissolution of aerosol particles in the apical liquid volume was the rate-limiting step for the overall absorption rate. The study also showed that the absorption rates of the tested formulations were similar after aerosolization and deposition in a multistage liquid impinger, which simulates more realistically the detachment of drug crystals from the carrier lactose and their aerodynamic particle size-dependent deposition in the respiratory tract following inhalation from a dry powder inhaler. Based on the results of their comprehensive studies, the authors made the following conclusions [70].

- Air interface deposition of soluble drug particles on pulmonary epithelial cell cultures yields higher transport rates and better reflects the situation *in vivo* than liquid interface deposition.
- Application by a simple insufflator device or liquid conditions may suffice as long as aerodynamic properties of the aerosol particles are not critical.
- To address aerodynamic properties, more sophisticated set ups such as a modified multistage liquid impinger should be used.
- Air interface deposition on pulmonary cell culture models offers a way to simulate the most important peculiarity of aerosol drug delivery: absorption of a relatively high metered dose after deposition on an only slightly wet epithelial surface.
- This approach may provide important extra information both for the assessment of pulmonary permeability of active pharmacological ingredients, as well as regarding the safety and efficacy of new excipients.

Solubilization of drugs in surfactant may affect the residence time of a drug within the lung and it is believed that surfactants in the lung increase the total amount of drug in solution by a factor of 3 to over 10 [71,72]. Based on this fact, many dissolution studies are conducted with simulated lung fluid (SLF). For example, SLF and modified SLF (mSLF) containing 0.02% (w/v) of dipalmitoylphosphatidylcholine (DPPC) were used for dissolution study of micronized bulk hydrocortisone using two different dissolution methods [72]. In the study, the authors concluded that the dissolution media containing DPPC (mSLF) can be used to predict the solubility and solubilization process of inhalation formulations with very low aqueous solubility because DPPC increases the wettability of hydrophobic drugs, and prevents aggregation, thus allowing an enhanced dissolution rate [72].

There is no doubt that the current trend in *in vitro* respiratory drug absorption investigations using tissue cultures is moving towards a more realistic simulation of *in vivo* particle deposition, active ingredient dissolution and subsequent absorption of dissolved drug. Another parameter thought to affect respiratory drug absorption *in vivo* is microgravity [73]. dos Santos *et al.* designed a cell culture/diffusion chamber system specifically to accommodate epithelial cell layers in a three-dimensional clinostat that allows epithelial permeability to be measured under microgravity conditions *in vitro* with minimum alteration to established cell culture techniques [74]. The objective of their work was to develop a diffusion chamber in which respiratory epithelial cell layers can be grown at an air interface under modeled microgravity conditions in the clinostat and into which cell layers can be inserted to permit drug absorption. In the study, microgravity was modeled in the three-dimensional clinostat by continuous random changes of orientation, relative to gravity's vector.

5. *In vivo* respiratory dosing methods

Particle deposition and distribution patterns within the lung and therapeutic efficacy of aerosols are very complex issues and depend strongly on the dynamic interactions of three classes of parameters: aerosol (aerosol formulation, the drug itself as an active pharmaceutical ingredient, the delivery device, etc.); respiratory tract (anatomy, geometry, breathing pattern: flow rate, tidal volume, etc.); and the patient (age, gender, disease status, etc.) [75]. Based on several factors that affect drug deposition in the lung, several methods have been used for dosing experimental animals during *in vivo* studies. Some commonly used methods include passive inhalation, forced instillation or direct intratracheal administration, head-only or nose-only method, and aerosol deposition using devices such as nebulizers, face masks, micro-spray and puffers [64]. Essentially, drugs can be administered to experimental animals by passive inhalation or directly to the lung in both a liquid or powder form [76]. Each of these approaches has peculiar advantages and disadvantages. The pioneer intratracheal instillation method was described by Schanker's studies [77]. Although the method yielded much needed information on drug disappearance from the lung, it is invasive and requires killing many animals for tissue sampling [75]. Evolution of the method involving cathetization has helped to improve the method and reduce the number of animals that are killed. During experiments, drug instillation may be carried out manually by hand or by the use of devices. Despite the approach that is used, this method results in regional drug spreading in the lung and poor reproducibility, as drug spreading depends on the volume of administration [77-80]. For this reason, the method is not favored by many scientists. Methods that involve passive inhalation of aerosolized drugs in awake and normally breathing animals are popular. Such approaches include a whole body exposure system and head-only or nose-only exposure systems [75]. According to Fernandes and Vanbever [76], although instillation of liquids into the lung is unpopular, this method is the preferred approach, especially when a precise control of the dose is needed because of direct drug administration into the trachea. Furthermore, when liquid instillation is done by means of orotracheal access using micro-spray devices designed to achieve more uniform distribution within the lung, use of a breath-controlled dosing unit, or a nebulization catheter, efficient and reproducible data can be obtained consistently with the instillation method [81-83]. Instillation is easy, quick and relatively inexpensive to use, and requires a small amount of drug for efficient administration. On the other hand, aerosol delivery is expensive, very inefficient and technically challenging, and it is very difficult to quantify precisely the delivered dose; however, the method is the most physiologic approach as it provides an even distribution of test drug in the lung [84].

For respiratory delivery of powders, insufflators are often needed. Various powder insufflation devices [76,81] have been developed, although most require tracheal access via tracheotomy and use 3 – 5 ml of compressed air to launch 0.2 – 5.0 mg of powder from the devices into the lung.

6. *In vivo* respiratory aerosol deposition and drug absorption screening methods

Particle size distribution in the aerosol and regional deposition within the lung play an important role in determining lung drug disposition. Therefore, it is important to use accurate methods to determine both regional and total lung aerosol deposition. Recent developments in techniques of radionuclide imaging have enabled quantification of whole as well as regional lung deposition in large and small animal models [76]. Several methods including gamma scintigraphy, single-photon-emission computed tomography (SPECT), positron-emission tomography (PET) and aerosol bolus methods have been used to determine regional particle deposition. Another important parameter for assessing drug disposition in the lung is total lung deposition, which depends

on the type of aerosol (supramicron aerosols, ultrafine aerosols, polydisperse aerosols, hygroscopic aerosols). Following aerosol deposition, drugs in the aerosol particles dissolve within the liquid film of the lung before absorption. In this section, important methods for screening regional and total particle deposition, as well as inhaled drug absorption in the lung, are highlighted [85].

6.1 Regional deposition assessment methods

Human lung deposition data may be generated using radionuclide imaging techniques or appropriate pharmacokinetic methods, and can act as a 'bridge' by which a seamless transition can be made between *in vitro* testing in the laboratory and efficacy: safety testing in the clinic [86]. Effective deposition of pharmaceutical aerosols in the peripheral lung, which can be confirmed relatively easily by gamma-scintigraphy, was considered as a surrogate of pulmonary bioavailability. Although this may be true for small molecules with favorable biopharmaceutical properties (i.e., good solubility in the pulmonary lining fluids and good permeability across cellular membranes), rapid and complete absorption across the air–blood barrier cannot be taken for granted for all pharmacologically active agents, especially not for macromolecular biopharmaceuticals such as peptides, proteins, or DNA/RNA-based drugs [87]. Two-dimensional gamma camera (planar gamma camera) is the most widely used imaging technique in pharmaceutical research [86]. The method enables lung deposition to be quantified accurately for any inhaled drug product, and also provides data on regional deposition by dividing the lungs into a series of zones, primarily representing airways of different sizes [85]. It provides direct information about the quantity (percentage of the dose or mass of drug) and site of deposition of the drug in the lung after inhalation [88]. However, the planar gamma camera provides only two-dimensional projection of the distribution and is thus very limited in distinguishing the distribution among airway regions and relating deposition to three-dimensional lung structure [87]. In planar images, the actual lung volumes projected on each planar compartment vary widely, and each compartment cannot be related to specific anatomic regions. Within these limitations, planar scintigraphy has been used to describe relative deposition between different lung compartments [89].

6.2 *In vivo* total lung deposition assessment methods

Although specific sites of deposition may not be obtained by conventional two-dimensional lung imaging, recent advances in three-dimensional imaging techniques such as SPECT show great potential for measuring local dose of particles in specific sites in the lung [88]. SPECT and PET provide accurate and highly specific discrimination between central and peripheral particle deposition in the three-dimensional intrapulmonary deposition pattern [87]. 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 three-dimensional distribution of radioactivity within the lungs [90]. In SPECT, 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 [76]. The disadvantages of this technique include the extra time to complete a scan and labeling of the drug of interest [88].

PET imaging involves specific positron-emitting radionuclides such as ^{11}C, which can be chemically incorporated into the structure of the drug molecule [86]. Although radionuclide imaging techniques can provide informative data, drawbacks include the cost of the equipment and the technical challenge of drug radio-labeling [76]. Three-dimensional imaging techniques (e.g., SPECT and PET) have the potential to give more detailed data on regional lung deposition, but are at present more expensive, use higher radiation doses, and are less well validated than two-dimensional (planar) imaging [91].

6.3 Respiratory drug absorption assessment methods using pharmacokinetic and pharmacodynamic methods

The therapeutic efficiency of respiratory aerosol is proportional to the dose deposited at the target site within the lung and the local bioavailability, rather than the systemic bioavailability after absorption, is pertinent as it reflects the efficacy of drugs that act directly in the lung [88,92]. Pharmacokinetic (PK) methods, using the urinary excretion of salbutamol after inhalation, have been shown to differentiate between the relative amounts of drug that are either delivered to the lungs or swallowed. As suggested by Hindle and Chrystyn [93], the 30 min urinary recovery of salbutamol, an index of the relative systemic bioavailability of salbutamol following inhalation, can be used to compare the lung deposition of nebulized systems. Also, the urinary 24 h recovery of salbutamol and its metabolite, an index of the relative systemic bioavailability of salbutamol following inhalation, can be used to compare the delivery of nebulized drug to the systemic circulation. Although pharmacokinetic studies can be used to demonstrate the level of systemic exposure, pharmacodynamic (PD) and clinical end point studies are used to demonstrate local action [94]. In essence, the pharmacokinetic method gives an indication of the total drug delivery to the systemic circulation via the pulmonary and oral routes and hence is useful when comparing the systemic delivery between different inhalation methods and delivery devices. A pharmacokinetic parameter such as relative bioavailability has a linear relationship with dose and correlates with clinical response [95-97].

Data generated from respiratory PD studies are the key parameters for demonstrating the effectiveness of respiratory drugs and serves as the backbone for clinical trials [85,87]. Efficacy and toxicity data obtained by means of PK-PD methods often assist in establishing proof-of-concept in efficacy and optimization of respiratory formulations [98].

7. Correlation between *in vivo* and *in vitro* methods

Accurate prediction of respiratory tract deposition is important in developing pharmaceutical aerosols for drug delivery [99]. *In vitro in vivo* correlations (IVIVCs) have the potential to serve as surrogates for *in vivo* bioequivalence studies, support biowaivers, and so reduce the number of *in vivo* studies undertaken during development; they can help to provide a greater understanding of the formulation and processing variables influencing clinical quality attributes and ensure the consistent clinical performance of different batches of manufactured drug product [100].

Correlations between *in vitro* measures of aerosol quality and indices of relative lung bioavailability have been demonstrated successfully [101-103]. Nevertheless, as suggested by Finlay and Martin [99] and de Matas *et al.* [104], publications that reported the correlation focused primarily on linear relationships between respirable dose from a nebulizer, fine particle dose from a dry powder inhaler and pharmacokinetic indices of relative lung bioavailability. The ability of linear regression equations to predict lung deposition in individual subjects for different formulations is likely to be limited by unexplained inter-subject variability observed in studies of this type. It has been shown that complex relationships exist between particle size, lung deposition and efficacy. It is also known that numerous product, physiological and patient factors can influence drug deposition, release, and the subsequent safety and efficacy of inhaled medicines [99,103]. Artificial neural networks (ANNs) have the potential and range of applications to overcome some of the problems associated with classical IVIVC, such as the provision of an *a priori* specification of the regression equation, and have been used successfully for predictive IVIVC delivery of salbutamol by nebulizer and DPIs [99,103]. Although the ANN model has been shown to be useful, the approach was constrained by the limited size of the database, and future evaluation of more comprehensive data sets could potentially lead to further improvements in the model predictability, particularly across different clinical studies [99].

The correlation between *in vitro* tissue culture studies with *in vivo* particle deposition and drug absorption data is more challenging. Well-designed *in vitro* tissue culture studies that integrate aerosol particle deposition on cells are continuously being developed, and in the near future predictive models will be developed. A study conducted by Warheit *et al.* [105] to forecast lung hazards following inhalation exposures to nanoparticles illustrates the challenges expected in such studies. In their study, the authors concluded that the implementation of the current cell culture methodologies tested in their study did not accurately forecast the pulmonary hazards associated with *in vivo* exposures to zinc oxide nanoparticles. Although the scope of this review article is limited to *in vitro* and *in vivo* methods, the importance of *ex vivo* models including lung perfusion may be found in some recent review articles [64,75].

8. Conclusion

Particle size and respiratory aerosol deposition in the lung are two major factors that determine drug absorption and disposition in the lung. Although *in vivo* drug deposition is a very complicated process, research has shown that *in vivo* drug deposition and absorption can be investigated using both *in vitro* and *in vivo* methods. With proper validation and careful experimentation, these new methods discussed in this review can be used for preclinical drug screening and quality control/quality assurance during the respiratory drug manufacturing process.

9. Expert opinion

The lung is a fairly complex organ with bifurcations that narrow the airways progressively towards the alveolar sac. Despite its complex anatomical features, it has formidable enzymatic and physical barriers (phagocytosis, mucociliary clearance), responsible for limiting particle entry and possible uptake by the organ. The anatomical and physiological nature of the organ makes it very challenging to deposit drugs deep enough within the lung for absorption to take place. During early stages of drug development, several *in vitro* and *in vivo* methods have been used to screen aerosol particle characteristics, drug deposition pattern and subsequent absorption. Each method has unique advantages and disadvantages that must be taken into account for experimental method selection, data interpretation and extrapolation to humans.

In vitro approaches involving particle size characterization using impaction and optical methods have been shown to be useful in determining aerosol part sizes and size distribution. Data from these methods are very useful during product development and quality control provided the methods are carefully validated. *In vitro* methods using tissue culture methods are particularly useful for mechanistic purposes, especially for defining the mechanisms of drug absorption by means of passive diffusion or active transport. Recently, tissue culture methods that integrate particle deposition and *in vitro* drug dissolution in a thin layer of liquid have been reported. These evolving new methods that attempt to mimic *in vivo* drug delivery in cell culture will eventually help in correlating *in vitro* data with *in vivo* particle deposition, drug absorption and efficacy. At the moment, *in vitro* methods complement *in vivo* data. *In vitro* results are also a very useful data source for mathematical modeling used for simulating *in vivo* respiratory drug deposition, absorption and disposition.

In vivo methods, especially scintigraphy, SPECT and PET, are used for screening regional and total particle deposition in the lung. Although the scintigraphic method is limited in application because of the nature of two-dimensional images acquired with the method, SPECT and PET are more useful for acquiring three-dimensional images and are therefore more relevant for determining total lung particle deposition. It is important to note that particle deposition within the

lung is not synonymous with therapeutic effectiveness. Pharmacokinetic and pharmacodynamic methods remain the principal approaches for investigating the extent of drug absorption after inhalation and the therapeutic effectiveness of the drug.

Declaration of interest

The authors state no conflict of interest and have received no payment in preparation of this manuscript.

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