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T. Sécher, E. Bodier-Montagutelli, A. Guillon, N. Heuzé-Vourc'h

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Sécher T[1,2], Bodier-Montagutelli E[1,2,3], Guillon A[1,2,4], Heuzé-Vourc'h N[1,2]

1. INSERM, Research Center for Respiratory Diseases, U1100, Tours, France
2. University of Tours, Tours, France
3. CHRU de Tours, Pharmacy Department, Tours, France
4. CHRU de Tours, Critical Care Department, Tours, France

Corresponding Author:
Dr Heuzé-Vourc'h Nathalie
INSERM, Centre d'Etude des Pathologies Respiratoires, U1100
Université François Rabelais de Tours
10 Boulevard Tonnellé
F-37032 Tours, France
nathalie.vourch@med.univ-tours.fr
phone: +33 2 47 36 62 37; fax : +33 2 47 36 60 46

Abstract

Nonclinical studies are fundamental for the development of inhaled drugs, as for any drug product, and for successful translation to clinical practice. They include *in silico*, *in vitro, ex vivo* and *in vivo* studies and are intended to provide a comprehensive understanding of the inhaled drug beneficial and detrimental effects. To date, animal models cannot be circumvented during drug development programs, acting as surrogates of humans to predict inhaled drug response, fate and toxicity. Herein, we review the animal models used during the different development stages of inhaled pharmaceuticals and biopharmaceuticals, highlighting their strengths and limitations.

Keywords: animal models, inhalation, pharmacodynamics, pharmacokinetics, toxicokinetics

Declaration of interest: NHV receives consulting fees from Cynbiose Respiratory, a CRO that develops and proposes animal models of respiratory diseases, including infectious pathologies.

Abbreviations

FDA: Food and Drug Administration; EMA: European Medicines Agency; PK: pharmacokinetics; PD: pharmacodynamics; MDI: metered dose inhaler; DPI: dry powder inhaler; CF: cystic fibrosis; COPD: chronic obstructive pulmonary disease; NHP: non-human primate; VAP: ventilator-associated pneumonia; ATB: antibiotic; MIC: minimal inhibitory concentration; RDS: respiratory distress syndrome; Ab: antibody; TSLP: thymic stromal lymphopoietin; PAP: pulmonary alveolar proteinosis; RSV: respiratory syncytial virus; ADME: absorption, distribution, metabolism and elimination; AUC: area under curve; CHMP: Committee for Medicinal Products for Human Use; IVIVC: *in vitro-in vivo* correlation; CYP: cytochrome P450; DBS: dried blood spot; BAL: bronchoalveolar lavage; ELF: epithelial lining fluid; OECD: Organization for Economic Cooperation and Development; ICAPPP: International Council on Animal Protection in Pharmaceutical Programs; FIH: first in human; GLP: good laboratory practices; MMAD: mass median aerodynamic diameter; EU: European Union; REACH: Registration, Evaluation and Authorization of Chemicals.

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Introduction

Like any new chemical entities (referred to in this review as pharmaceuticals) or biotechnology-derived products (referred to as biopharmaceuticals), the development of inhaled drugs from laboratory concept to regulatory approval by the Food and Drug Administration (FDA), the European Medicines Agency (EMA) or other regulatory institutions, is generally multi-step, long and costly. To guarantee drug quality and patient safety, the FDA and the EMA provide guidelines on the investigations that should be carried out on medicinal products. Such guidelines cover nonclinical - pharmacodynamics (PD), pharmacokinetics (PK) and toxicity studies - and clinical studies, some of them being done according to Good Laboratory Practice (GLP) regulations. Well-designed preclinical (or nonclinical) studies are foundational and critical to successful pharmaceutical or biopharmaceutical development programs. The design of nonclinical studies varies greatly and depends on drug characteristics, in particular their nature (pharmaceutical or biopharmaceutical). In some cases, deviations from guidelines may be necessary and accepted by regulatory agencies as long as they are scientifically relevant. Nonclinical studies include *in vitro* assays (using for instance human samples), *in silico* modeling and *in vivo* assessments of the drug's pharmacological properties and safety profile. Although regulatory agencies encourage alternative methods to animal testing for nonclinical studies to comply better with the principle of the 3Rs (replacement, reduction and refinement), animal models remain helpful and/or mandatory for certain phases [1, 2]. During preclinical development, animal models are used as surrogates of humans to predict dose-response, drug toxicity, PK and PD. In the case of inhaled pharmaceuticals, such preclinical evaluations should also be carried out in conditions set to be the most clinically relevant. In this context, the inhalation route is probably the most challenging route of administration in animal models when it comes to extrapolate nonclinical results to humans. Fortunately, many inhaled drugs are repurposed or repositioned drugs for pulmonary delivery, thereby limiting the use of animals to necessary pharmacological studies – usually safety and PK studies - and leading to a more rapid product development [3].

As mentioned in the FDA guidance, inhaled drugs are sorted as follows: (i) inhaled drug products "intended for delivery to the lungs for local and/or systemic effects and administered by metered dose inhalers (MDI), dry powder inhalers (DPI) or nebulizers" and (ii) nasal drug products "applied to the nasal cavity for local and/or systemic effects" [4, 5].

In clinical practice, inhaled drug products can be administered in the respiratory tract as dry or liquid aerosols. In the case of dry aerosols, drugs are formulated as powders, which are de-agglomerated and dispersed into fine inhalable particles by a DPI. Conversely, liquid aerosols consist in fine droplets produced from liquid drug formulations (solutions or suspensions), which can be aerosolized as single puffs (a few microliters) by MDIs through

the release of a compressed gas, or continuously with nebulizers, which can aerosolize several milliliters of drug product. Three types of nebulizers can be used for such purposes: jet nebulizers (which use a compressed gas source), ultrasonic nebulizers (droplet generation resulting from the application of ultrasounds) or mesh nebulizers (in which droplets are produced by passing the drug liquid through a calibrated mesh) [6]. In animal models, - as reviewed elsewhere in the manuscript - experimental drugs can also be administered in the airways either as bulk or aerosolized liquids, or as powders for which aerosolization is mandatory.

In this review, we considered only orally inhaled drug products for the treatment of respiratory diseases and described the experimental models used for regulatory studies leading to their approval. It is noteworthy that the majority of inhaled drugs has been used for decades and approved long ago when regulatory guidelines were not well documented. Despite ours efforts to review animal models described in the literature and regulatory application files for marketed inhaled pharmaceuticals, including models for PD, PK and toxicity studies, we may have missed some information. We also chose to highlight the requirements and particularities of animal models used for the preclinical development of biopharmaceuticals, since they are the fastest growing class of therapeutics and have a tremendous opportunity to benefit to patients with respiratory diseases.

This review describes, first, the experimental models for PD of inhaled pharmaceuticals and biopharmaceuticals, highlighting their relevance to mimic some features of human pathophysiology. Next, it addresses strengths and weaknesses of animal models for PK assessments used to define effective and safe dosage regimens for first-in-human (FIH) studies. Finally, it discusses experimental models and regulatory guidelines used for toxicity assessment of inhaled pharmaceuticals.

1. Models for the pharmacodynamics of inhaled pharmaceuticals and biopharmaceuticals

1.1 Fundamentals of pharmacodynamics for inhaled drug products

PD is defined as the response of the body to a drug and refers to as “What the drug does to the body”. PD studies are intended to investigate “the mode of action and/or effects of a substance in relation to its desired therapeutic target”, as referred by the EMA [7]. Together with PK, they help explain the relationship between drug concentration at the site of action and the resulting effects (regarding intensity and time course), whether they are desirable or adverse, and select the dose for nonclinical and clinical studies. They often do not comply with GLP regulations.

In the case of inhaled pharmaceuticals, the site of action can be either the respiratory tract (topical action) or elsewhere (systemic effects). For most inhaled drugs, the concentration at the site of action usually determines the intensity of drug's effect and is directly correlated to aerosol deposition, making aerosol delivery critical to achieve the expected response. Inhaled drug effects can also be modified by pathological conditions and can be studied using several experimental models – *in vitro* or *in vivo* – trying to reproduce, at least to a certain extent, the clinical situations in which it is intended to be used.

Many inhaled products being repurposed or repositioned for pulmonary delivery [3], their efficacy has not always been established after inhalation delivery. For example, primary PD of asthma and chronic obstructive pulmonary disease (COPD) drugs (including muscarinic receptor antagonists, β2-adrenergic receptor agonists and corticosteroids) were studied *in vitro* in genetically-modified cell-lines or *ex vivo* in airway explants from both human and animal origin to assess drug affinity for its cognate receptor. Such drugs can also be associated into combinations of two or three products, each of them being typically characterized alone in preclinical models and used as a single therapy in the clinics. Usually, such combinations are intended for the treatment of multifactorial complex diseases, such as asthma and COPD (see Table 1), modulate the activity of different targets, improve the selectivity/efficacy of single molecules and decrease side-effects and toxicity [8]. So far, additional *in vivo* experiments are not required for the nonclinical development of combinations containing approved compounds. This may be questionable since many combinations emerge from the clinical practice and were not designed as such from scratch. Thus, our understanding of the molecular interactions of these combined drugs may be incomplete.

Looking closely to the animal models used for the development of inhaled pharmaceutics (see Table 1), several of them can be distinguished for PD studies.

1.2 Model for the development of inhaled pharmaceuticals in airways inflammation and bronchial hyperresponsiveness – importance of the guinea pig

Guinea pig is a species of rodents, often used as a surrogate for humans in inhalation studies owing to its anatomical and physiological resemblance with human lungs [9, 10] and its capability to cough and sneeze. In addition, it is the species that most closely matches the human pharmacology of M2-, M3-muscarinic and β2-adrenergic receptors [11] and the autonomic innervation of airway smooth muscles [12, 13]. As a result, contractile and relaxant agonists of airway smooth muscles display similar potency and efficacy in guinea pigs and humans, leading to an extensive use in the preclinical assessment of inhaled bronchodilators and corticosteroids in COPD and asthma (see Table 1, [14, 15]). For the two drug classes currently available (namely muscarinic receptor antagonists and β2-adrenergic

receptor agonists, which have both demonstrated their clinical efficacy with limited side effects [16]), guinea pigs were used for preclinical mimicking of bronchoconstriction induced mainly by methacholine or acetylcholine (see Table 1).

The first experimental model of asthma in guinea pigs was established in 1937 [17], taking advantage of their hypersensitivity response to allergens (*i.e.* smooth muscle contraction,…), mainly attributable to histamine acting on histamine H1 receptors, which are similarly expressed in guinea pigs and humans [14]. Afterwards, guinea pigs were used as an asthma model to test the preclinical efficacy of bronchodilators or corticosteroids against histamine-induced lung inflammation (see Table 1). The major drawback of guinea pigs comes from their prominent tendency to develop a lung axon reflex (activation of sensitive nerves in the airways, subsequently inducing characteristic features of asthma in response to challenge) or insult, which is not observed in humans [18-20]. In addition, the limited diversity of specific reagents, the paucity of wild-type strains and the lack of genetically-modified strains limit their use for in-depth comprehension of the molecular mechanisms associated with allergy or COPD. Finally, guinea pigs have long-time gestation and give birth to few offspring as compared to other rodent species [14].

1.3 Models for the development of inhaled pharmaceuticals for viral lung infections – importance of the ferret

Ferrets were used to assess the preclinical efficacy of the inhaled antiviral molecules laninamivir [21], zanamivir [22, 23] and sialidase [24], owing to their high sensitivity to human strains of influenza virus [25]. This is probably due to the expression of α2-6-linked terminal N-acetylneuramidic sialic acid in the respiratory tract [26]. This is the not the case for mice, which require ~~time-consuming~~ viral adaptation. Following infection, ferrets exhibit clinical symptoms observed in humans, including sneezing, fever, nasal discharge and inflammation [27]. In comparison, rodents or non-human primates (NHPs) (depending on the strain) do not recapitulate all these features, thereby limiting extrapolation of results to humans in which mitigation of symptoms is one of the primary endpoints in clinical trials. In addition, influenza virus can be transmitted between ferrets, recapitulating an essential and “natural” characteristic of the human disease. Finally, the upper and lower respiratory tracts of ferrets present similarities with humans.

Despite the pathophysiological relevance of the ferret model towards influenza infection, ferrets are not perfect because of the limited availability of animal suppliers, genetic heterogeneity due to outbred background, high husbandry costs, lack of immunological reagents and genetically-modified strains. Additionally, they may not be appropriate to assess the efficacy of (inhaled) anti-flu antibodies: indeed, the human Fc displayed a very short half-life in ferrets as compared to mice [28]. Additionally, inhalation delivery (under

liquid or dry forms) is not easy to perform in ferrets. That is presumably why investigational studies used dissolved compounds and the intranasal route for administration, which is questionable [29]. Future directions include (i) the development of genetically-modified strains benefiting from the recent publication of the ferret genome [30] and the development of the CRISPR-CAS technology, (ii) a reduction of costs associated with ferrets use considering the positioning of ferrets for antiviral drug development since 2008 and a worldwide effort to understand pandemic influenza viruses and (iii) a refining of the route of administration for antiviral drugs with the development of specific devices which may mimic better human inhalation [31].

1.4 Models for inhaled pharmaceuticals against bacterial lung infections

Inhaled therapeutics are developed to fight bacterial lung infections mainly for two respiratory conditions: ventilator-associated pneumonia (VAP) and cystic fibrosis (CF). VAP is a nosocomial pneumonia that complicates the clinical course of mechanically-ventilated patients in intensive care units (ICUs). CF is characterized by recurrent bronchial obstruction due to mucus accumulation, bacterial airways infections and persistent inflammation. *Pseudomonas aeruginosa* is one of the predominant pathogens responsible of lung infection in both clinical situations. The eradication of *P. aeruginosa* has become increasingly difficult due to its remarkable capacity to resist to intravenous antibiotics (ATBs). Therefore, inhaled ATBs have been developed as alternative strategies.

Preclinical testing of inhaled ATBs is usually carried out on bacterial cultures (including laboratory, clinical, and drug-resistant isolates) rather than in animal models, as is the case for systemically-delivered ATBs. The results help identify the best lead with the lowest minimal inhibitory concentration (MIC), to decipher the mechanism of action (bactericidal *vs* bacteriostatic) and resistance potency [32, 33].

Experimental models of nosocomial pneumonia have been set up in a wide range of laboratory species. However, animals should be anesthetized and ventilated in their physiologic prone position for several days and handled in experimental conditions reproducing the ICU environment to model VAP accurately. Specific ventilator settings are critical for the performance of nebulization and should be close to those used in ventilated patients. For these reasons, a mechanically-ventilated anesthetized piglet model has been developed, combining prolonged mechanical ventilation with massive bronchial inoculation of highly concentrated pathogens [34]. Bactericidal efficiencies of aerosolized and intravenous ATBs have been compared in this model [35]. More generally, mechanically-ventilated anesthetized piglets are largely used to model aerosol delivery during invasive mechanical ventilation in humans (adults).

The development of inhaled anti-*P. aeruginosa* ATBs to treat respiratory infections in CF patients mainly relied on clinical studies along with preclinical studies in rodents. Most murine experiments used a simple one-hit strategy, far from representing CF features. It usually consisted in a unique administration of a large bacterial inoculum, giving rise to pulmonary or extra-pulmonary infections depending on the injection site – intratracheal/intranasal delivery resulted in pneumonia while systemic injection (thigh or intravenous) led to upper respiratory tract infection or sepsis (see Table 1). Besides, those models are characterized by acute infections - with a high 1 to 3-day mortality - which is far from *P. aeruginosa* colonization or chronic infection encountered in CF patients. Another pitfall of wild-type mice is the lack of bronchial submucosal glands and consequently mucus over-production as observed in CF patients, which may represent a substantial barrier for inhaled therapy [36]. Thus, several other species have been considered for CF studies, including the pig [37]. Anatomically, pigs have submucosal glands, relevant target tissues for CF pathogenesis, which spreads along cartilaginous airways into the pulmonary parenchyma. Hopefully, genetically-modified CF pigs will be helpful to accelerate translational research and optimize inhaled antimicrobials in this pathological context.

1.5 Models for inhaled drugs in respiratory distress syndrome

Surfactant deficiency leads alveoli to collapse during normal tidal breathing, resulting in generalized atelectasis and ultimately respiratory failure. Several animal-derived surfactants are marketed and delivered topically through intratracheal instillation to prevent and treat respiratory distress syndromes (RDS) in preterm infants with surfactant deficiency due to lung immaturity (also known as Hyaline Membrane Disease). Interestingly, the first attempt to deliver surfactant phospholipids (dipalmitoylphosphatidylcholine) in the airways was achieved by aerosolization in infants at risk of developing a RDS and failed to demonstrate any beneficial effect of surfactants. In the 1970s, Enhorning and Robertson successfully developed a rabbit model of surfactant replacement therapy. Preterm rabbits were supplemented intratracheally with animal-derived surfactants from mature rabbits, paving the way for the development of effective surfactant treatments and further evaluation in newborn infants [38]. Up to now, a wide variety of surfactant products, both extracts derived from animals and synthetic (protein-free) surfactants, have been developed and tested either in premature rabbits, like the princeps study, or neonate lambs reproducing deficiency of pulmonary surfactants. It is noteworthy that several late-stage clinical trials are currently testing inhalation of (aerosolized) surfactant for RDS treatment (NCT03058666, NCT02294630, NCT03582930).

1.6 Requirement of specific and dedicated animal models for inhaled biopharmaceuticals

Biopharmaceuticals are defined as “therapeutic materials produced using biological means, including recombinant DNA technology”, and comprise protein therapeutics (antibodies (Abs) and other proteins), vaccines, antisens, RNAi technologies and molecular technologies. Presently, there is only one marketed inhaled biopharmaceutical– dornase alpha, a recombinant enzyme used as a mucolytic in CF patients. Despite the paucity of inhaled biopharmaceuticals on the market, we chose to address inhaled biopharmaceuticals in this review since several inhaled molecules are in clinical development and biopharmaceuticals represent approximately 25% of the global drug market (2010-2017 data [39]) and have a tremendous opportunity to benefit to patients with unmet needs in respiratory medicine [40]. With the exception of a few vaccines, all inhaled biopharmaceuticals in clinical trials are recombinant protein therapeutics (see Table 2). ~~It is not surprising, as protein therapeutics make up two thirds of the marketed biopharmaceuticals.~~ One of the main features of protein therapeutics is their high specificity for their molecular target, on the one hand limiting their off-target activity and on the other hand allowing restricted species cross-reactivity. Standard rodent models are often inadequate to assess PD of lead protein therapeutics, thereby necessitating the use of surrogate molecules for mechanistic studies. For instance, the efficacy of CSJ117, an anti-human-thymic stromal lymphopoietin (TSLP) antibody fragment, has never been preclinically tested before its evaluation by inhalation in adults with mild atopic asthma. The proposed mechanism of action of CSJ117 relies in part on a study with an anti-mouse TSLP receptor Ab tested in a murine model of asthma (see Table 2), complemented by *in vitro* assays. Indeed, the EMA encourages the use of *in vitro* assays to assess the biological activity of biotechnology-derived pharmaceutics. As stated by the EMA, cellular assays can be used “to predict specific aspects of *in vivo* activity” and “assist in the selection of an appropriate animal species for further *in vivo* pharmacology” [41].

As shown in Table 1 and Table 2, the animal models used in PD studies are diversified, including large mammals and genetically-modified rodent models. Genetic manipulations result in knock-out, knock-in and transgenic animals and are largely spread out in mice [42]. The advances in genetic engineering technologies and the ability to generate animal models with genetic alterations linked to human diseases made the mouse a popular model for biopharmaceuticals. For instance, several preclinical studies for sargramostim/molgramostim were conducted in GM-CSF knock-out mice with a surrogate drug - a recombinant murine GM-CSF. The animals spontaneously developed pathological features resembling the human disease pulmonary alveolar proteinosis (PAP). Humanized mice, in which murine genes are replaced with their respective human orthologues, are also interesting for the development of inhaled protein therapeutics. For example, the activity of PRS-060/AZD1402

- an anticalin antagonist of human IL-4 receptor alpha - was tested in a human cell line and its efficacy was assessed in humanized mice expressing human IL4Ra and IL-4/13. It is noteworthy that mice with a reconstituted human immune system would certainly be valuable for inhaled protein therapeutics since respiratory diseases are often associated with immune dysfunction.

Larger animal models have also been used for inhaled protein therapeutics, usually in parallel of a rodent model as they allow the delivery in conditions closer to inhalation in humans. Notably, ALX-0171 (trimeric) Nanobody™ was given by inhalation in newborn lambs, using a face-mask, to assess the therapeutic response against respiratory syncytial virus (RSV) infection. The use of NHP models is also on the rise with biopharmaceuticals, especially for the nonclinical development of therapeutic Abs, as they are often the only relevant species based on biological and immunological considerations. The anti-asthmatic action of both CDP7766 and pitrakinra was evaluated in an allergic asthma model developed in NHPs and induced by *Ascaris suum*; proteins were delivered through aerosol delivery (by nebulization). Although NHPs are relevant for both biopharmaceuticals and aerosol delivery, the increasing ethical pressure of the society makes them more and more difficult to use for scientific purposes and drug development.

2. Normal lung models for pharmacokinetic studies of inhaled drugs

2.1 ADME considerations for inhaled pharmaceuticals

PK studies are carried out to support studies on clinical efficacy and define effective and safe dosage regimens, and are not always performed according to GLP standards. PK speaks of “What the body does to the drug” and the principal objectives are to describe the absorption, distribution, metabolism and excretion of the active substance (sometime referred to as ADME), which is a requirement of regulatory agencies around the world.

Absorption refers to both the rate and extent in which inhaled pharmaceuticals are available systemically. Depending on their physicochemical properties, drugs are absorbed either by transcellular absorption or through the tight junctions (paracellular absorption). Absorption is usually determined from plasma/blood concentration-time curve data following. Systemic exposure of inhaled drugs follows absorption, either directly into the pulmonary circulation, or through the gastrointestinal tract (after ingestion of oropharyngeal deposits and drug removed from the lung by mucociliary clearance). Although new *in vitro* screening methods (organs-on-a-chip) are developed and may be relevant, animal models remain the standard to analyse absorption. Distribution refers to the transfer of inhaled drugs to other relevant body fluids and tissues not belonging to the respiratory system, usually following absorption. For instance, the central nervous system may be directly reached by aerosol particles through the nose-to-brain pathway [43]. Metabolism refers to the study of metabolites that

can be formed within the lungs after aerosol drug deposition. For example, ciclesonide (an inhaled corticosteroid) is pharmacologically inactive; its transformation into a sole pharmacologically active metabolite, desisobutyryl-ciclesonide, occurs in the lung [44]. Excretion refers to the internal elimination rates (*e.g.* renal and hepatic eliminations) of the inhaled drug and its active metabolites.

Looking at the regulatory applications of inhaled pharmaceuticals, ADME studies are not always conducted during inhaled drug development. Indeed, drugs are often repurposed for inhalation and there are already available data in the literature regarding ADME (for other routes). The distribution, metabolism and excretion of inhaled drugs are not expected to change after their absorption into the systemic circulation, thereby data obtained with other routes can be considered relevant for inhalation delivery. For instance, the distribution of inhaled tobramycin powder was not evaluated but relied on results obtained with ^{14}C-labelled tobramycin injected subcutaneously in rats. In contrast, drug absorption and lung exposure, which depend on the route of administration, are usually investigated following inhalation. The assessment of systemic exposure can constitute an integral part of the toxicity studies, named toxicokinetics. As referred by the EMA, toxicokinetics is defined as “the generation of pharmacokinetic data, either as an integral component in the conduct of nonclinical toxicity studies, or in specially designed supportive studies, in order to assess systemic exposure”. Toxicokinetics is carried out in a relevant animal model, with the pharmaceuticals administered by the intended route to describe systemic exposure. Measurements consist in blood sampling (plasma or whole blood or serum) to measure the concentration of inhaled drugs and/or relevant metabolites. As an example, absorption, systemic and lung exposures of inhaled tobramycin powder were investigated in the serum and lung tissues of rats and dogs in single- and multiple-dose toxicity studies. Because systemic exposure of inhaled drugs also follows gastrointestinal absorption, one cannot rule out that investigations in animal models following parenteral or oral administration provide somehow relevant data to inhalation exposure. For instance, ADME of colistimethate sodium or colistin sulphate was investigated in various animal models following oral and intravenous administration when developed as a powder for inhalation. For biopharmaceuticals, such as protein therapeutics, animal ADME studies may not be relevant for biological and immunological reasons. For example, the elimination of human alpha1-proteinase inhibitor in animals was not representative of the situation in humans due to protein immunogenicity; the lack of data was regarded as acceptable by the EMA/Committee for Medicinal Products for Human Use (CHMP).

2.2 Studying lung-specific pharmacokinetic processes in experimental models

For inhaled drugs, it is also important to consider what happens before systemic absorption (see Figure 1), which comprises (i) drug deposition into the respiratory tract as only a fraction of the loaded drug effectively reaches the lungs; (ii) drug dissolution in the lung fluids; (iii) clearance through the mucociliary system in the conducting airways (and then transfer to the gastrointestinal tract by swallowing) and macrophages in the alveolar space; and (iv) pulmonary tissue absorption/retention and pulmonary metabolism .

2.2.1 Deposition of inhaled drugs in experimental models

Only a fraction of the dose loaded in the device gets deposited in the lungs, while the remaining fraction may be lost in the device, spread in the environment, or deposited in the oro-nasopharynx and then swallowed. In humans, inhaled drug deposition is influenced by aerosol particle size, drug formulation, inhalation flow, device performances and disease-related factors.

Regarding the relevance of animal models for this purpose, lung exposure is ultimately dictated by the respective deposition/clearance balance of each species, which may limit transposition of the results even when comparing an identical inhalation setup. Indeed, aerosol deposition ~~in the lungs~~ is also conditioned by animal breathing patterns, ventilation parameters and airways anatomy, among others. Differences in respiratory physiology and body size ~~thus~~ lead to heterogeneities among mammals (including humans). Interspecies comparisons of lung anatomy have been reviewed in more details elsewhere [6]. As a result, aerosol particles display different deposition profiles in mammals, in terms of both preferential deposition sites and deposition rates. Particle size is an important determinant, as particles under 0.5 µm are unlikely to deposit in the lungs of rodents, dogs or humans [45]. The available literature reports roughly similar overall deposition of 1-5 µm particles in nose-breathing humans, monkeys and dogs, while lower deposition levels were retrieved in rats [46]. Conversely, nasal and tracheobronchial deposition are exaggerated in rats as compared to humans [47]. ~~Pulmonary deposition seems to follow similar trends, with dogs and monkeys being close to humans, and rats displaying lower deposition rates.~~ Finally, optimal particle size for aerosol deposition differs among species: while maximal relative lung deposition is obtained with particles of about 1 µm for rats, 2-4 µm is more suitable for human lungs [45].

In practice, several methods are available to deliver pharmaceuticals/biopharmaceuticals in the lungs of laboratory animals (see Section 3.2.2). Non-invasive aerosol delivery methods - in spontaneously breathing animals - result in important extra-pulmonary deposition and absorption. Indeed, because experimental animal models are mostly nose-breathing, a large amount of the inhaled aerosol deposits in the nasal cavity. ~~This fraction may be transmitted to the GI compartment, where it can be absorbed into the bloodstream.~~ It is noteworthy that

some aerosol procedures in non-anesthetized animals may induce stresses ~~require restraint and tight-fitting seals around the animals~~, leading to ~~stress-induced~~ modifications of the respiratory and circulatory physiology (*e.g.* increased respiratory rate and blood flow) that can be confounding factors for PK analyses. Thus, it is worth getting the animals accustomed to the procedure [48].

Alternatively, oro- or intra-tracheal administration consists in delivering a suspension of drug droplets/particles directly into the trachea using a spray/aerosol and bypasses the oro-nasopharynx [49-53]. To a PK point of view, ~~direct intratracheal administration~~ it reduces extra-pulmonary absorption [54, 55]. The rate of lung absorption of the inhaled active substance could thus be quantified and compared to an equivalent intravenous dose [53]. Despite its advantages (*e.g.* limited amount of drug required, precise dosing and bypassing of the nasal structures), this method of administration lacks representativeness towards human aerosol administration. Moreover, this technique is invasive and the anaesthesia may impact biological functions (altered mucociliary clearance, surfactant destabilization, increase of alveolar epithelial permeability) [56-58]. It is also noteworthy that oro-/intra-tracheal delivery may induce lung lesions, modifying the passage of the drug from the lungs to the systemic circulation and therefore biasing blood-derived PK [59]. ~~Hence, intratracheal administration is rather adapted to early discovery steps (*e.g.* PD studies), compound screening or mechanistic toxicity studies.~~

Aerosol delivery during invasive mechanical ventilation in animal models is particularly relevant to a translational point of view for PK studies, as the oropharynx is bypassed and sealed by the endotracheal tube. For example, mechanically ventilated pigs have been commonly used to study inhaled ATBs PK [34, 60]. In this experimental setting, one can consider that ATB concentration in the blood is appropriate to predict the inhaled drug behaviour in the lung compartment. Ventilator-assisted aerosol inhalation has been applied with success in rodents, reproducing a lung-physiological drug deposition profile [61, 62]. A step ahead is to study PK in animal models of lung infections, as lung lesions may critically influence the bioavailability calculated from the plasma/serum concentration time profiles [53, 63, 64].

Finally, it is important to consider the impact of the device intended for animal experiments on drug stability - especially if the case of biopharmaceuticals. For instance, protein therapeutics may aggregate during the aerosolization process [65, 66] and such aggregates may display a different behaviour compared to the native protein and induce antidrug antibody (ADA), ultimately impairing PK profiles [67-69].

~~Finally, the assessment of inhaled drug deposition in experimental models poorly takes into consideration the patient/inhaler interaction. How and when the patient uses the inhaler or nebulizer is crucial for drug delivery to the lung, still this factor is absent in animal~~

~~experiments. Non-adherence to therapy has been identified as the main factor for lack of disease control in asthma: a large majority of patients fulfilled the criteria of suboptimal adherence, which was defined as <80% of prescriptions filled. Next, the inhalers, *per se*, are critical for deposition performance. A wide range of inhaler devices and systems are available in the clinics. In recent years, several technical innovations have improved device portability, materials of manufacture, breath actuation, the interface with the patient, combination therapies, dose tracking, etc. These recent developments are critical for the overall therapy performance but can hardly be integrated in the practical design of experimental protocols. For *in vitro-in vivo* correlation (IVIVC), the absence of these two last parameters (*i.e.* patient inhaler interaction and inhaler types and properties) is an important limiting factor.~~

2.2.2 Dissolution of inhaled pharmaceuticals in experimental models

After deposition, aerosol particles, especially solid ones, are expected to dissolve into the epithelial lining fluids, which consist in (i) the airway lining fluid and the mucus in the conducting airways and (ii) the alveolar lining fluid and the pulmonary surfactant in the alveolar region [70]. Drug dissolution in the lining fluids directly impacts drug absorption/lung retention and depends on its physicochemical properties, formulation and pathophysiological factors of the patient. Molecular size and hydrophobicity are determinant for dissolution and transport across lining fluids. Besides, drug formulation can be optimized to enhance or reduce the dissolution rate for short-acting drugs, or to extend lung retention and effect of duration, respectively. The mucus layer is a gel whose thickness varies along the conducting airways [71]; it usually acts as a barrier towards drugs. Mucus Diffusion diffusion of macromolecules, such as biopharmaceuticals, through the mucus partly depends on their size and molecular weight [72]. In pathological conditions, mucus dehydration and thickening may impair drug dissolution and transport/absorption [73]. Surfactant may have a dual role facilitating the dissolution of inhaled drugs (e.g. glucocorticosteroids) but increasing their clearance when used as an excipient (e.g. tobramycin) [74-76]. Dissolution of inhaled pharmaceuticals is mostly investigated in vitro and ex vivo, in isolated and perfused lungs from animals. It is noteworthy that some experimental models (mice, rabbits) lack bronchial submucosal glands, making them less relevant to assess the impact of inhaled drug dissolution/transport across lining fluids [77, 78].

2.2.3 Inhaled drug clearance by the mucociliary transport system and alveolar macrophages

Once aerosol particles are deposited, lung-specific clearance mechanisms may modify final lung exposure to the drug as well as systemic absorption from the lungs (see Figure 1). The

mechanisms involved partly depend on the particle characteristics (*i.e.* their solubility in airway fluids, protein binding capacity, physical size, etc.), but also on their site of deposition (which correlates with particle size).

In the upper airways (nasopharynx and trachea), particles may be removed by mechanical efforts such as sneezing, coughing and eventually swallowing. Besides, mucociliary transport plays a major role in particle removal from the airways. Interestingly, the literature does not report major differences in tracheobronchial clearance among mammals, in spite of heterogeneous mucus velocities: in small animals, lower mucus velocity may be compensated by shorter airways [77]. However, the lack of bronchial submucosal glands in small rodents (mice, rabbits) has to be considered [77].

Conversely, alveolar clearance is more variable across species, partly due to heterogeneous number, size and distribution of alveolar macrophages, which are involved in the uptake and elimination of insoluble particles [77, 79]. Distribution of alveolar macrophages are different in rats, dogs, baboons, and humans ; superior number of alveolar macrophages are observed in human lungs [79] , which may result in faster alveolar clearance [79]. Finally, enzymatic clearance is also a source of heterogeneity across mammals, through the differential expression of cytochromes P450 (CYP) and phase II enzymes by several cell types in the airways [80]. For instance, the human lung expresses low levels of CYP as compared to the liver, whereas the levels are similar in rats (despite variations in isoenzymes) [81].

2.2.4 Pulmonary tissue absorption/retention and metabolism

The rate and extent of lung uptake depends on drug physicochemical properties - lipophilicity, ionization, affinity for tissue macromolecules and pulmonary tissue partitioning coefficient - and patient characteristics [70]. Drugs distribute in the lungs through passive diffusion, and then interact with cells, partitioning into membranes and subcellular organelles. Any inter- and intra-species differences that may significantly affect pulmonary tissue absorption should be taken into consideration when selecting an animal model. For example, absorption of lipid-insoluble drugs is different from one species to another. On the contrary, lipid-soluble drugs are similarly absorbed into the blood circulation of numerous animal species [46, 82]. Young animals are often used for nonclinical studies, but pulmonary absorption is age-dependent [83]. For instance, the half-time rate of mannitol pulmonary absorption is 32±2 min in neonatal rats, *versus* 60±6 min in adults [83]. In addition, the duration of lung development differs markedly between mice and humans, limiting simple correction with age [84]. It is noteworthy that interactions of the inhaled drug with endogenous proteins - which expression may substantially vary across the different respiratory biological matrices - should also be taken into consideration to select the most relevant animal model.

Although lungs contain drug-metabolizing enzymes, they are not expected to play a major role in drug metabolism compared to other organs (gastrointestinal tract, liver). However, proteolytic activity in lung lining fluids may be critical for inhaled proteins/peptides, especially during pathological conditions in which the protease-inhibitor balance is impaired. Isolated and perfused lungs from animals (usually rats and rabbits) have been used to investigate the dissolution, absorption and metabolism of many inhaled molecules [85], and provide relevant biochemical data to support inhaled (bio)pharmaceutical PK [85, 86]. For example, polyhydroxyethylaspartamide absorption was investigated in isolated, perfused and ventilated rat lungs and displayed an active, energy- and temperature-dependent absorption mechanism [87]. Similarly, Tronde *et al.* used isolated and perfused rat lungs to show rapid absorption of an opioid tetrapeptide agonist across the air-blood barrier, associated with limited metabolism in the lungs [88]. Recently, isolated and perfused rat lungs were used to build a mathematical model to predict pulmonary absorption [89]. Techniques for lung perfusion have been described in various species (guinea pigs, rats, rabbits) and isolated lungs provide several assets, such as a careful control of the lung function, easy drug delivery, sampling and mass-balance determination. Drug administration is often done by instillation for simplicity, but *ex vivo* lungs can be combined with relevant aerosol delivery methods [62, 90]. A major shortcoming is the rapid deterioration of isolated and perfused lungs (4-5 hours), which is not compatible to investigate slow biological processes.

2.3 Sampling in animal models to investigate pharmacokinetics of inhaled pharmaceuticals

2.3.1 Blood sampling

PK of inhaled pharmaceutical are atypical by the fact that blood is not upstream of the site of drug action (as for oral dosage) but downstream. ~~Blood/plasma/serum drug concentrations cannot evaluate bioequivalence at the site of action but rather expected side effects on other organs. As referred by the EMA, toxicokinetics is defined as "the generation of pharmacokinetic data, either as an integral component in the conduct of nonclinical toxicity studies, or in specially designed supportive studies, in order to assess systemic exposure". Toxicokinetics is carried out in a relevant animal model, with the pharmaceuticals administered by the intended route to describe systemic exposure. Measurements consist in blood sampling (plasma or whole blood or serum) to measure the concentration of inhaled drugs and/or relevant metabolites.~~ Blood samples are adequate to evaluate absorption and systemic exposure after inhalation. As mentioned earlier, these important parameters of PK may be evaluated during toxicity studies, thereby constituting toxicokinetics.

~~However,~~ Some particular features - inherent to the animal models or dependent on the technology used for inhalation - are important to consider when analysing blood samples to

determine PK parameters. Sampling time points depend on the tested substance, the route of administration and the species to avoid any interference with normal physiology. In general, aerosol dosing usually requires much longer sampling than is required for intravenous studies. Mice are not adapted to undertake large and/or multiple blood samplings from lung dosimetry studies, because it is difficult to obtain enough blood from the same animal. To overcome this issue, composite sampling is often used, where blood is collected at different time points from different animals across a time course, providing sufficient volume/time points for PK analysis. As an alternative, serial sampling can be used for Dried Blood Spot (DBS) analysis, which requires a small amount of blood dried as a "spot" on sample cards [91]. In larger animals, multiple blood sampling is not an issue, allowing profiling of systemic serum/plasma levels in one animal.

2.3.2 Sampling in the lung compartment

Lung exposure is an important PK parameter for inhaled pharmaceuticals. It can be estimated by computing blood concentrations in mathematical models. However, given the multifaceted absorption process that follows drug inhalation and the rise of biopharmaceuticals with complex PK profiles, blood is not always a suitable surrogate compartment to predict lung exposure/PK. Samples for PK studies can be collected directly in the respiratory system in the form of lung tissue, bronchoalveolar lavage (BAL) fluid, or *in situ* microdialysis. BAL is a procedure consisting in injecting fluid into the lungs and then collecting it by re-aspiration, for examination (i.e. measurement of drug concentration in the airways and epithelial lining fluid). One should be careful of the distinct meaning of BAL in animal experimentation and medicine. In small animals, BAL is usually performed on the whole respiratory tract, *via* tracheal catheterization; in humans, this procedure concerns distal portions of the lungs (*via* bronchoscopy) and thus examines alveolar regions. In this prospect, larger animal species better reproduce the human BAL procedure [92]. Usually, BAL provides data for a single time point in one animal because the procedure itself interferes with local drug concentrations and thus PK parameters. Indeed, the injection of saline solution into the lungs induces an artificial dilution of all components. To circumvent this potential bias, it is essential to use an endogenous biomarker that diffuses passively through the different tissue compartments as an internal control [93]. Alternatively, drug concentration can be measured directly in the lung tissue, harvested during animal necropsy and requires careful lung exsanguination to avoid data bias~~, resection, and homogenization of lung tissue, followed by drug extraction with appropriate solvents~~. For peptide/protein-based therapies, attention should be paid to their lability due to the presence of endogenous proteinases. Finally, iterative tissue sampling implies the use of numerous animals and is thus ethically questionable.

In vivo microdialysis is a semi-invasive sampling technique that has been used to study drug PK in various tissues [94-97], including the lungs [95, 98, 99]. Because it does not alter homeostasis, this technique allows repeated measurements from the same animal of soluble and/or unbound drug in the interstitial space,. For instance, continuous PK measurements of various drugs have been successfully performed in the lungs with this technique [95, 98]. Interestingly, lung microdialysis of inhaled biopharmaceuticals has recently been shown feasible [99, 100].

It is also important to consider some inter-species differences that may skew PK analyses. First of all, anatomical characteristics of the lungs considerably differ among mammals (*e.g.* branching scheme of the tracheobronchial system, number and size of the pulmonary acini, lobulation of the lungs) and clearly matter for translating results across species [77]. Any inter- and intra-species differences that may significantly affect the PK profile should be taken into consideration for animal model selection.

3 Models for toxicological studies of inhaled pharmaceuticals

This section provides an overview of the issues associated with toxicology studies for inhaled pharmaceutics, which should be performed according to GLP standards. In addition to similar principles shared with other routes of administration, inhaled drug toxicology presents some specific concerns. Indeed, the generation, delivery and dosing of inhaled materials are of particular importance and require highly controlled experimental conditions. In this context, the use of animal models for inhalation toxicity testing has been historically based on guidelines. Several *in vivo* models are available for inhalation toxicity testing, although the toxicological database for inhaled materials is still limited [101, 102]; besides, the use of animals remains questionable due to interspecies differences in the anatomy, histology and physiology of the respiratory tract [52]. In this context, the use of *in vitro/ex vivo* systems may reduce the unreliability associated with extrapolation across species. However, they may not be able to accurately reproduce the complexity of the inhalation process, which is crucial for a relevant IVIVC regarding drug toxicity.

3.1 Regulatory guidelines for the toxicity assessment of inhaled drugs

The purpose of toxicology studies during pharmaceutical development is to predict, as reliably as possible, the safety profiles of these products once delivered to patients. There are a limited number of specific guidelines for inhalation toxicity studies; study designs are usually similar to those of non-inhaled pharmaceuticals [103] and are not necessarily relevant for this peculiar route of administration. Recently, the Organisation for Economic Cooperation and Development (OECD) published test guidelines regarding inhalation and histopathological analyses for acute, 28-day repeated dose and 90-day sub-chronic dose

studies [104-107]. More recently, the pulmonary division of the FDA provided a draft document, which indicates that toxicology packages should include short-term studies (2 to 4 weeks) in two species (comprising one non-rodent) and a 6-month chronical study in the most appropriate species, followed by a carcinogenicity study if proliferative or preneoplastic changes have been observed. Study design should include air control, vehicle control, and complete formulation groups. For repurposed marketed products and according to the amount of preexisting information on systemic toxicity, the FDA recommends a 28-day study in rodent and non-rodent species followed by a 6-month study in the most appropriate species [108].

It is worth noticing that the International Council on Animal Protection in Pharmaceutical Programs (ICAPPP) has urged to re-examine the value of routine testing in a second species, as the use of one species is actually regarded as acceptable when clearly justified.

3.2 Inherent specificities of toxicity studies for inhaled drugs

3.2.1 Choice and use of animal

Animal species selection, as mentioned above, is governed by guideline recommendations but also needs to fulfill specific requirements associated with inhalation itself. Animals should be small enough to ease handling, housing and exposure in sufficient number favoring readily statistical analysis. However, they should be big enough to permit all valuable measurements to assess the toxicity of the inhaled material. While additional factors like animal strain, age, health status as well as housing conditions are universal, reproducible test atmosphere and acclimatization periods to the dosing device are specific for this route and may influence toxicity assessment.

The quest for the best animal model to assess toxicology of inhaled pharmaceuticals appears to be unwise according to the intricate parameters related to this route of administration. As underlined below, specific issues of species susceptibilities, exposure methods, formulation, dose administered and end-points to monitor make the selection of the most relevant animal model difficult.

3.2.2 Aerosol generation and administration

Regulatory safety assessments most commonly recommend administration to animal species using aerosol inhalation, to be as close as possible to human conditions. In practice, aerosol inhalation is commonly achieved by dispersing the aerosol into a controlled stream of air, which is ultimately inhaled through an interface. Different types of apparatus, compatible with the most common inhalers (*i.e.* nebulizers, DPIs and MDIs) can be used [109] and can broadly be sorted as whole-body or nose-only exposing devices. In whole-body systems, entire animals are exposed to the tested atmosphere; they tend to see their use decrease,

due to considerable drug losses and massive fur deposition, leading to drug ingestion through self-grooming [47]. As a result, lung exposure is generally weak as reported to the drug concentration in the breathed air, in addition to unwanted systemic delivery. Targeting the animal breathing zone - to reduce the amount of drug needed, increase lung deposition yields and obtain experimental conditions closer to human inhalation - is feasible with nose-only exposure systems for rodents and lagomorphs and helmets, face masks or oro-pharyngeal tubes for non-rodent species (*e.g.* dogs, sheep, and monkeys) [109, 110]. The selection of the inhalation device may be also guided by drug specificities: oro/endo-tracheal tubes may be relevant for limited-amount drugs, or in case of reported deleterious nasal irritancy [47]. This type of equipment offers interesting possibilities in terms of breathing patterns: anesthetized dogs can be placed in spontaneous breathing or under mechanical ventilation, continuously or with breath holds (to mimic the human breathing pattern during aerosol inhalation) [110]. However, a large part of the upper airways is bypassed, which may limit prediction of human toxicity in this area. Nose-only devices and face masks are better adapted for a global exposition of the respiratory tract. However, this mode of administration may lead to overestimations of nose exposure, due to differences in respiratory physiology between laboratory species and humans [52, 109]. Overall, consideration should be given to the similarities between the device, the dosing apparatus and the formulation used in preclinical studies and those used in humans in order to make relevant interspecies correlations. Indeed, excipients (including propellants used in MDIs) may modify the behavior of the aerosolized drug in terms of particle size, mucoadhesion, dissolution in lung fluids, or safety profile. Thus, guidance recommends to perform GLP toxicity studies with formulations as close as possible to FIH formulations [45].

~~In practice, aerosol inhalation in animals requires the use of an aerosol generator plugged on an inhalation interface placed between the inhaler and the animal.~~ Aerosol generators frequently differ between discovery, preclinical and FIH studies, due to the specific constraints and desirable characteristics related to each development step. For instance, good laboratory practices (GLP) toxicology studies typically require inhalers compatible with the available exposure systems (see below), whereas in a clinical perspective, patient compliance, portability and cost-effectiveness of the device may be of more importance [6]. As a consequence, different aerosol devices may be used throughout the development process, to fit both the advancement of the drug product (*e.g.* formulation work) and the characteristics of the aerosol administration setup. As an example, for the development of montelukast, a leukotriene receptor antagonist, different types of DPIs were used for PD, toxicology and FIH studies [110]. In such cases, every device should be thoroughly characterized to ensure the predictability of preclinical safety data to humans. ~~Indeed, aerosol characteristics are major determinants of lung deposition, and thus of potential~~

~~toxicity.~~ Another strategy would consist in employing the same device for preclinical and clinical studies ~~This implies either to use a marketed device, or to design the final inhaler at an early stage in the drug development program. In this case, safety evaluations could be carried out with the final device, which might,~~ to virtually circumvent any data bridging issues [110]. But, other transposability concerns may emerge from this approach. For example, the device should be suitable with available animal exposure systems and should guarantee a similar deposition rate in animals (especially with regards to aerosol particle size).

3.2.3 Dosimetry / Lung exposition to pharmaceutical aerosols

To assess in a reliable way the toxicity of inhaled drugs, the evaluation of lung exposure to the investigated aerosol is of particular importance: either the drug is expected to remain (and act) in the lung, or a systemic passage is expected. In both cases, prediction of its toxicity requires to sufficiently expose the lung tissue to the aerosol. In practice, defining and achieving relevant doses in the lungs - *i.e.* sufficiently high to generate toxicity, but also somewhat meaningful regarding the final treatment regimen - remain challenging. More specifically, the peculiarity of the inhaled route lies in the fact that it is almost impossible to accurately determine the dose effectively delivered to the lung.

As mentioned earlier, lung exposition depends on the interplay between deposition and clearance mechanisms, which may not be representative of human physiology in all common laboratory species. This parameter is of paramount importance to control the delivered dose and further assess its toxicity. In animals, the pulmonary dose can be assessed by lung tissue excision and analysis, or by autoradiography following the inhalation of a radiolabeled aerosol [45]. Still, these quite burdensome procedures are often applied on a small number of animals, in single-exposure satellite studies. For larger cohorts, the delivered dose is commonly estimated by the following mathematical formula [47]: Delivered dose = RMV x C x D x F / BW

where RMV = Respiratory Minute Volume, C = drug concentration in the test atmosphere, F = inhaled fraction, D = daily duration of exposure and BW = animal body weight.

However, technical characteristics of the drug product (pharmaceutical form, amount of material available) and of the experimental setup (animal model and inhalation technology) significantly limit the doses achievable in practice [47]. In addition, other technical parameters related to the inhalation apparatus might lower these maximal doses. Based on these considerations, one could propose to adapt tunable parameters of the equation to adjust the delivered dose [111, 112] [113].~~. Among these parameters, concentration and duration of exposure are controlled and known, as well as body weight. The RMV, which can be measured in some specific experimental setups, is commonly estimated based on the body weight however, this approach does not allow time-related corrections, which may be~~

~~relevant in the case of drug-dependent irritancy of the respiratory tract~~ Finally, the inhaled fraction is often considered to be 100%. Additionally, the FDA recommends estimating the pulmonary dose by using deposition factors of 10% and 25% for rodents and non-rodents respectively, depending on the aerosol mass median aerodynamic diameter (MMAD). Another approach is to maintain a MMAD < 4 µm, as recommended for chronic and sub-chronic studies, and to use the maximum achievable dose [110]. Exposure times could also be occasionally extended, to increase the inhaled dose. Aerosol characteristics also depend on the type of aerosol generator (nebulizer, MDI, etc.), which bear singular technical limitations. As a result, attainable doses in these conditions are usually less than 1 mg/kg in rodents with MDIs [47]. In the case of inhaled drugs for which systemic exposure is suspected, it may be necessary to complement inhalation exposure with systemic administration to attain substantial toxicity [114], as it has been done for the development of salmeterol [115].These technical limitations make the limit dose difficult to define in preclinical safety assessments; for low-toxicity drugs, this might be of particular importance, as current toxicity study designs might not allow to reach doses that cause assessable toxicities.

3.2.4 Assessment of respiratory tract toxicity

According to the characteristics of the inhalation route, inhaled therapeutics toxicity must be assessed both locally and systemically [116]. Indeed, local effects reflect the initial (and expected) deposition site of the drug inside the airways, while systemic effects may occur following oral absorption and/or translocation of the drug from the respiratory tract to the blood system. This later condition, which is usually associated with systemically-acting drugs, necessitates the evaluation of systemic toxicity, which can be predicted using toxicokinetic methods. Therefore, when making extrapolation of toxicological data for inhaled drugs, PK data are critical [114].

To assess the local toxicity of inhaled drugs, classical lung histopathological analyses should be performed ~~They include a full necropsy at the end of the exposure time, with organ weight measurement and gross lesions quantification in both proximal and distal regions of the airways~~ [45] and include microscopic examination of cellular infiltration, epithelial damages, necrosis, hyperplasia and fibrosis. Histopathological analyses can be complemented with BAL to document and follow the extent of an inflammatory response and analyze pulmonary functions.

The interpretation of inhaled pharmaceuticals toxicological data from animal models must be done with caution due to species specificities. In this field, large animals like dogs or monkeys provide more relevant results than rats. Indeed, rats usually exhibit squamous metaplasia of the larynx at the end of the dosing period, but this response has been

demonstrated to be non-specific and reversible [117]. In addition, rats are prone to a decrease in their number of alveolar macrophages, but, in the absence of other indicators of inflammation, this response is considered as non-adverse [118]. Larger animals may be more relevant in terms of toxicity markers, even though specific species sensitivities cannot be totally ruled out. For instance, toxicity profiling of therapeutic surfactant included a 2-week study in dogs. Intratracheal instillation caused respiratory distress, which was attributed to an exacerbated sensitivity of dogs to volume and/or viscosity of solutions [119]. This exemplifies the importance of testing at least two different animal species during toxicological assessment of inhaled drugs.

Finally, the inhaled drug may have confounding toxic effects on different animal species. It was the case for dornase alpha, a human recombinant DNase I. Inhalation by rats and monkeys induced the production of antidrug-antibodies, whereas in humans, it was not the case [120]. ~~In addition,~~ Histological evidences of lung inflammation were also observed, both concurring to the observation of a non-specific foreign body reaction instead of a toxic reaction due to inhaled dornase alpha in animals. The presence of excipients in drug formulations should also be addressed in toxicological analyses, especially if they are unconventional. This was the case for the development of Afrezza™, an inhaled form of human insulin (intended for a systemic action), which formulation included fumaryl diketopiperazine. Due to the novelty of this excipient, regulatory agencies asked for a 2-year inhalation carcinogenicity study in rats together with a 6-month subcutaneous study in transgenic mice [121]. A limited number of excipients being currently approved for inhalation, this situation may be encountered for other drugs, especially if their physicochemical properties require the use of novel excipients.

4. Relevance of animal models for inhaled (bio)pharmaceutical development

Nonclinical studies are the pillars for the development of human (inhaled) drug products and successful translation to clinical practice. Although regulatory agencies encourage avoiding unnecessary animal use, animal models (*in vivo* or *ex vivo*) cannot be totally circumvented, so far, in the pharmacological package of inhaled drug products. Many animal species have been used for the non-clinical development of inhaled drugs and discussed in this review. Each animal model displays its own features, advantages and limitations, but none of them is able to mimic perfectly the human respiratory function and its pathophysiology. Based on our experience and the literature [52, 77, 122, 123], we attempted to summarize and score – in Figure 2 and Table 3 - animal models used in PD, PK and toxicology studies for inhaled pharmaceuticals/biopharmaceuticals development. Although a meticulous comparative analysis of the physiologic, technical and experimental features is always required for selecting the most appropriate animal model during drug development [52], it becomes

critical and difficult for inhaled drugs, as the inhalation route is one of the most challenging points when trying to extrapolate animal results to predict inhaled drug behavior in humans.

When looking carefully regulatory application files of inhaled drug products, the most striking point is the paucity of nonclinical studies using mice, while this species is very popular in biomedical research, often considered as a Jack-of-all-trades. For instance, mice models have been widely used in laboratories to dissect molecular mechanisms involved in the pathogenesis of asthma or to identify novel targets [124, 125]. However, for PD studies, the anti-inflammatory response to corticosteroid was mostly investigated in guinea pigs, a model of non-pulmonary delayed type hypersensitivity reaction induced by antigen sensitization (see Table 1). This may be attributed to the fact that mice predominantly express β1-receptors in the airway smooth muscle, lack mediators of hypersensitivity, one feature of allergic asthma, while guinea pigs are more relevant models [126, 127]. Similarly, mice are often neglected for toxicokinetic studies, most probably because they are not appropriate for repeated sampling. Recent advances in genetic engineering technologies and ease of development have rendered mice highly versatile and adapted for the development of inhaled biopharmaceuticals.

Historically, rats were more popular than mice in the study of obstructive pulmonary diseases. Indeed, their larger size and volumes of biological fluids (BAL, blood) are compatible with the measurement of pulmonary functions (airway hyperresponsiveness (AHR), acute responses to allergen), and investigation of pulmonary inflammation and PK. A significant advantage of rats over mice is their proximity to humans with regard to lung mechanics (early- and late-phase response as well as AHR [128]). Like mice, their main drawback is due to their tendency of developing tolerance against challenged allergens after sensitization, thereby restraining development of chronic lung pathologic responses and late changes in lung structures (fibrosis, emphysema) as those observed in asthmatic or COPD patients. Their use has declined in favor of mice in recent years, probably due to the limited availability of genetically-modified rats.

Dogs respiratory frequency and tidal volume are close to human respiratory parameters. With the development of aerosol exposure methods mimicking human devices [129], dogs are often used as a non-rodent species for toxicological studies of inhaled drug products. ICH S7A guideline has established the use of conscious dog under non-stressed, physiological conditions has the preferred model for safety studies on the cardiovascular system [130].

Similarly to humans, sheep display several key features of human respiratory tract anatomy including size of the nasal cavity and the airways [131]. Consequently, lambs/sheep have been used as models for aerosol delivery using either mechanical ventilation for the

development of recombinant alpha antitrypsin [132] and surfactants [133] or face-mask aerosol exposure for the development of anti-RSV antibody [134].

Pigs are an interesting surrogate of humans due to anatomical, physiological and metabolic similarities and are considered as a translational model in pharmacological studies [135]. For instance, pigs are appropriate to investigate drug deposition in the lungs and inhaled drug ADME owing to resemblances to humans for respiratory parameters, numerous membrane transport and enzymes [136, 137]. The major limitation of pigs comes from their nose anatomy rendering aerosol administration poorly feasible under spontaneous breathing, their narrow mouth opening making intubation/intratracheal procedures difficult [138] and the financial issues associated with housing requirements/garbage elimination. It is noteworthy that pigs have human-similar lung tissue markers a size compatible with translational lung imaging. These features help exemplify why pigs were considered a leading species candidate for genetic manipulations of cystic fibrosis transmembrane conductance regulator CFTR [128].

Even if NHP are often considered as the gold standard model by the US FDA for biopharmaceutical development, they require extensive handling to minimize stress as well as anticipated gestures. Primates necessitate specialized equipment and techniques along with increased housing costs and ethical concerns.

5. Alternatives to animal models of inhaled pharmaceuticals

The guidance for industry regarding safety pharmacology studies notifies that the expected clinical route of administration should be used, if possible, during preclinical development. As highlighted in this review, the interspecies physiological, anatomical, cellular and biochemical differences in the respiratory tract along with the financial and labor costs complicate aerosol deposition and inhalation conditions mimicking (drug & device) across species (Figure 2). Thus, *in vitro* models such as anatomical models, impactor technology and mathematical, computational fluid dynamics models to predict aerosol deposition are helpful to bridge animal studies to the first clinical use.

Given the recent public and scientific publications questioning the translational value of animal models and the growing ethical pressure on animal use, it is important that researchers and industrials consider/develop alternative non-animal models to demonstrate drug scientific validity and to reduce their use [139, 140]. Although a complete replacement of animal testing does not appear very reasonable, there have been exciting advances in *in vitro* models of the pulmonary environment, notably (i) culture of epithelial cells at the air-liquid interface (ALI) recapitulating the cellular complexity of the lung epithelium in interaction with air in physiological conditions, (ii) (lung) organoids with tissue-like structures and (iii) the microfluidics lung-on-chip, which recreates a functional alveolar-capillary interface with

stretching forces mimicking the mechanical forces of breathing. They benefit from many advantages like a lack of concerns regarding cross-species correlation, ethical issues and economic constraints and can be supportive for many development stages. Even if *in vitro* methods for toxicity assessments of inhaled pharmaceuticals have not been officially accepted by regulatory authorities yet, they have benefited from substantial support by the regulatory framework of new European Union (EU) regulations regarding Registration, Evaluation and Authorization of Chemicals (REACH) and the OECD [141]. They have expanded scientific knowledge and speeded up the development of reliable alternative methods, which may be relevant for toxicity testing of the increasing number of inhaled therapeutic products [142]. The main limitation of standard cell culture approaches (nasal/lung epithelial cells (Calu3, Beas2B, 16HBE14o) and alveolar type II cells (A549, H441, hAELVi)) comes from their limited relevance regarding respiratory biology. Indeed, in their physiological environment, airway cells face the air with their apical side while they are supplied by nutrients from their basolateral side. The use of the ALI techniques allows growing epithelial cells in an environment that favors the differentiation program towards and airway phenotype. For respiratory cells, ALI conditions induce the secretion of mucus, surfactant, expression/function of cilia and the expression of tight-junction proteins (see [143, 144] for comprehensive review). These conditions can be used for the culture of both cell lines and primary cells from human biopsies. Refined 3D models may be set up [145] using artificial scaffolds to provide mechanical support for the cells, or co-culturing them with tissue-derived fibroblasts, immune (macrophages, dendritic cells) or endothelial cells. Interestingly, these models can be combined with novel inhalation exposure systems, which may offer new standards in the development of inhaled pharmaceuticals over the coming years [146]. Commercially available systems using gravitational (CULTEX®), impaction (VITROCELL®) or electrostatic (NAVETTA®) deposition can be coupled with human inhalers, further improving the physiological relevance of *in vitro* toxicology testing [147].These systems allow the direct application of aerosols onto cell surface more closely resembling the *in vivo* context and can be kept in culture for months allowing long-term toxicity assessments. Still, they are difficult to implement and require extensive efforts to become reliable and standardized enough to be used as predictive models [148]. No *in vitro* methods are for instance suitable for regulatory assessment. Howbeit, recent inputs from the academic, industrial and regulatory communities may help in standardizing and making recommendations that may ultimately help validate *in vitro* methods for toxicology assessment of inhaled pharmaceuticals [149].

Conclusions and future directions

To conclude, *"Essentially, all models are wrong, but some are useful"* [150]. This is particularly true for the models used for inhaled drug development. None of them fulfills all the criteria to mimic perfectly the human lungs and breathing pattern, thus requiring continuous reassessments in order to make them the most predictive of the clinical conditions. Thanks to the recent advances in cellular and delivery technologies, there is a fascinating outlook for the future of non-animal models in translational medicine and nonclinical assessment of inhaled drug products. In the end, the most important remains the selection of the most predictive model and both animal and non-animal models may be complementary to satisfy the recommendations for pharmacological studies.

Figure legends

Figure 1: Scheme describing the deposition & fate of inhaled (bio)pharmaceuticals. Based on Borghardt *et al.* [151].

Supplementary Figure 1: Identity cards of major animal models used for regulatory approval of inhaled drugs products summarizing their main advantages and limitations

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