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Oral inhalation for delivery of proteins and peptides to the lungs

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Graphical abstract

Highlights

Pulmonary delivery of biologics is studied for the treatment of obstructive lung diseases, acute lung injury, pulmonary arterial hypertension and idiopathic pulmonary fibrosis.

Aerosolisation of liquid formulations is the most common technique for pulmonary application.

In addition to the approved DNase product Pulmozyme®, AIR DNase, alpha1-proteinase inhibitor, recombinant neuraminidase, and heparin are the most advanced inhaled biologics.

Abstract

Oral inhalation is the preferred route for delivery of small molecules to the lungs, because high tissue levels can be achieved shortly after application. Biologics are mainly administered by intravenous injection but inhalation might be beneficial for the treatment of lung diseases (e.g. asthma). This review discusses biological and pharmaceutical challenges for delivery of biologics and describes promising candidates. Insufficient stability of the proteins during aerosolization and the biological environment of the lung are the main obstacles for pulmonary delivery of biologics. Novel nebulizers will improve delivery by inducing less shear stress and administration as dry powder appears suitable

for delivery of biologics. Other promising strategies include pegylation and development of antibody fragments, while carrier-encapsulated systems currently play no major role in pulmonary delivery of biologics for lung disease. While development of various biologics has been halted or has shown little effects, AIR DNase, alpha1-proteinase inhibitor, recombinant neuraminidase, and heparin are currently being evaluated in phase III trials. Several biologics are being tested for the treatment of coronavirus disease (COVID)-19, and it is expected that these trials will lead to improvements in pulmonary delivery of biologics.

Keywords: Inhalation, biologics, drug delivery, nebulizers, lung diseases, protein delivery, ARDS treatment

1. Introduction

Pulmonary delivery offers rapid absorption and, in general, higher blood levels than other non-invasive application routes. This rule applies mainly for small molecules but pulmonary delivery may be suitable to deliver biologics for local and systemic treatment. Lung diseases such as asthma, chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) are traditionally treated with small molecules administered by oral inhalation. Biologics may represent a better way to treat these diseases, as inflammatory processes could be treated more selectively and with less systemic effects than with inhaled corticosteroids due to the barrier between respiratory epithelium and capillary endothelium for macromolecules. This was demonstrated by instillation of antibodies against *Pseudomonas aeruginosa* in animal models. The inhaled antibodies reduced the permeability of the respiratory epithelium, while systemic infusion restored endothelial function [1]. Parenterally delivered interferon (IFN)- γ resulted in high serum levels, but low penetration into the bronchoalveolar lavage fluid (BALF), while the opposite was noted for inhaled IFN- γ [2]. The authors measured IFN- γ levels 1h and 24h after administration of one single dose and detected levels below threshold in BALF after subcutaneous injection and in blood after inhalation at both time points. In addition, only 5% of inhaled monoclonal antibodies directed against vascular endothelial growth factor and 11% against epithelial growth factor (cetuximab) were detected in mouse serum [3]. Higher efficacy of inhaled compared to injected biologics has also been reported for local administration. Inhalation of anti-IL-13 Fab fragments in a murine asthma model was more efficient than intravenous administration of the fragments [4]. The fully human AR711 antibody isolated from B-secreting cells of patients with convalescent coronavirus disease (COVID)-19 was effective after instillation in severe acute respiratory syndrome (SARS)-CoV-2 infected golden hamsters. Efficacy scaled up to the human situation would be 0.03 mg/kg compared to 3 g/kg by intravenous injection [5]. Despite these promising data, administration of biologics by oral

inhalation is not straightforward, as there are several biological challenges to overcome the protective mechanisms of the lung and pharmaceutical issues associated with biologic delivery. The challenges and potential solutions are summarized in Figure 1 and are addressed in this review.

2. Biological challenges

Biologics can act in the mucus layer, target cells of the respiratory epithelium or cross the respiratory epithelium to act in the lung parenchyma (e.g. smooth muscle cells). Further differences exist in terms of the region in the lung (small airways or alveoli), where they are intended to act. To illustrate these differences, the morphology of the airways is briefly described. Conducting airways, which transport the inhaled air to the regions, where gas exchange takes place, include the trachea and bronchi until the 16th generation (Fig. 2). The term “generation” describes the fact that the respiratory tract branches in an almost symmetric manner to form the bronchial tree and that at each bifurcation the number of generations increases by one. The parts of the respiratory tree from 17th generation onwards (referred to as the respiratory airways), including the respiratory bronchioles, alveolar ducts and alveolar sacs, perform CO₂ and oxygen exchange [6]. The general principle for particle deposition in the lungs also applies to biologics. Particles with aerodynamic diameter greater than 10 µm deposit in the extrathoracic region (nose, mouth, pharynx, larynx) and between 2 and 10 µm particles in the tracheobronchial tree [7]. There are several reviews dedicated to this topic for further information (for instance [8, 9]).

After deposition, biologics that act in the mucus (dornase, lonodelastat) or on pathogens in the lung can take effect. Other biologics need to cross the bronchial mucus layer or alveolar surfactant and the fluid covering the epithelial surface of the lung, usually called alveolar lining fluid (ALF) or epithelial lining fluid (ELF) to reach the target cells. The mucus layer of the conducting airways is between 5-55 µm thick and forms a mesh with hydrophobic and negatively charged sites [10]. The mucus properties are relevant to the drug delivery strategy. Enhancing mucus adhesion (e.g. by coating with chitosan) can increase bioavailability, while anti-adhesive properties (e.g. by binding to large hydrophilic molecules) allow better mucus permeation. In general, pulmonary mucus is not a significant barrier for macromolecules <10 nm (~500 kDa, [11]) [12]. However, if drug particles or protein aggregates remain in the mucus, they are transported from the deep lung to the pharynx (“mucociliary clearance”) by the cilia of the bronchial epithelial cells. Clearance is less effective when the diameter of the airways becomes smaller and the number of ciliated cells decreases. In contrast to the epithelium of the conducting airways, the respiratory epithelium of the alveoli is covered by a thin layer of pulmonary surfactant (0.07-0.3 µm thick, [10]) consisting of lipid-rich structures arranged as tubular myelin, bi- and multi lipid layers and an aqueous layer (termed hypophase).

Epithelial cells below the acellular layers (mucus, surfactant) are bronchial epithelial cells and goblet cells in the conductive airways and predominantly (96%) alveolar epithelial cells type 1 in the alveoles. Cells of the respiratory epithelium are the target for recombinant neuraminidase, solnatide, and BIO-11006. Other biologics (e.g. antibodies, cytokines, heparin, and activase) must also cross the epithelial layer. They can permeate the epithelium via transcytosis (through the cells, e.g. passive diffusion or carrier-mediated), paracellular (between the cells) and through large transitory pores in the epithelial layer. The optimal LogP for passive diffusion is 2-9, and peptides that have a LogP <0 rarely use this pathway [13]. Absorption via the paracellular route is restricted to molecules of certain size. Intercellular tight junctions of the respiratory epithelium have a cut-off of 0.5-0.9 nm, while the underlying endothelium allows the passage of molecules up to 6.5-7.5 nm in diameter [14]. Higher temperature (still in the physiological range) increase absorption [15]. Carrier/receptor-mediated transport exists for di- and tripeptides through proton-coupled peptide transporters (PEPT) [16] but it is unlikely to contribute greatly to most inhaled peptides. When peptides are ingested by pinocytosis or endocytosis, they usually are found in the lysosomes, where degradation is likely due to the high content in hydrolases [17]. This leads to their inactivation and requires development of strategies to prevent intracellular degradation. Fusion proteins, which are constructs between the protein of interest and the Fc receptor of immunoglobulins (Igs) are one possibility to prevent degradation. Human bronchial epithelial cells express the neonatal fragment crystallizable (Fc) receptor, also termed Brambell receptor or FcRn. It is a Major Histocompatibility Complex (MHC) I like molecule, that functions to protect immunoglobulin (Ig) G and albumin from catabolism, mediates IgG transport across epithelia and is involved in presentation of antigens by antigen-presenting cells [18]. The intracellular receptor is localised to early endosomes and binds IgG and albumin entering the cell by unspecific pinocytosis. After binding of IgG or albumin, the FcRn separates from the sorting endosomes to recycling endosomes, and the ligand is released into the extracellular space [19]. Animal data also showed 10-fold longer retention in the FcRn wildtype than in the knockout mice [20]. Biologics fused with the Fc part were able to cross the respiratory barrier by this route [21] but it seems unlikely that this mechanism contributes significantly to the systemic delivery of inhaled therapeutic monoclonal IgGs [22]. Transferrin-mediated transcytosis of Filgrastim (granulocyte colony stimulating factor, G-CSF) and transcytosis of albumin have been reported *in vitro* on rat alveolar epithelial cell monolayers [23, 24]. Type 1 and 2 alveolar epithelial cells have both clathrin- and caveolin-mediated endocytosis, but uptake by type 1 alveolar epithelial cells is mainly caveolin-mediated [25]. Unlike clathrin-mediated endocytosis, caveolin-mediated uptake does not involve transport to lysosomes with protein degradation. Caveolin-mediated uptake is therefore the preferred route for delivery of biologics. Encapsulation in nanoparticles and selectively targeting for caveolin-mediated uptake by coating with ligands for specific receptors has not been used for pulmonary delivery of biologics so far [26].

The 20-25 nm thick basement membrane with anionic charge is located under the respiratory epithelium, but is not a major barrier to passage of biologics (Fig. 2). Conversely, in the interstitium below the basement membrane biologics can bind to other macromolecules, be phagocytosed by interstitial macrophages or be transported into lymphatic vessels [27].

2.1. Metabolization and degradation of biologics in the healthy lung

In general, metabolic activity in the lung is lower than in the gastrointestinal epithelium and the liver, and inhaled drugs are subject to lower first pass effect. This difference is particularly dramatic for small molecules. For example, fluticasone propionate was metabolized 99% when taken orally and 0% when inhaled [28]. However, degradation of proteins and peptides also occurs in the respiratory tract. Small natural peptides (<3 kDa) are more affected than larger (6-500 kDa) proteins. It has been reported that the degradation of luteinizing hormone-releasing hormone by type 2 alveolar epithelial cells is higher than by the type 1 cells, suggesting that the former has higher protease activities [29]. The most abundant proteases in BALF from healthy individuals are cathepsin (Cat) D, CatH, angiotensin-converting enzyme (ACE), and dipeptidylpeptidase (DPP) IV [30]. Further, metalloproteinase (MMP) -2 in the hypophase and plasma membrane bound carboxypeptidase M of alveolar epithelial type 1 cells can influence the effect of inhaled proteins and peptides [31, 32].

In addition to epithelial cells, uptake may occur by alveolar macrophages (AMs), which perform functions of the unspecific immune system (e.g. phagocytosis and antimicrobial action) [33]. Cells recovered from BALF were mainly AMs (85%); other immune cells like lymphocytes and neutrophilic granulocytes represent a minority of 10% and 1-2%, respectively [27]. In addition to removal by uptake, secretion of peroxidases and proteases by the immune cells reduces protein absorption. However, degradation and uptake by AMs seem to play only a minor role, as more than 95% of proteins are absorbed intact from the lung periphery [15, 34]. This finding can be explained by the fact that macrophages preferentially take up proteins in denaturated form and/or as protein aggregates, but not intact proteins [35].

2.2. Disease-related changes

Diseases can cause alterations in bronchial diameter and alveolar structure (eg. bronchial constriction, emphysema). Furthermore, changes in mucus thickness and protease content are relevant for protein delivery. While degradation of insulin was found to be lower in diabetic than in healthy rats [36], a higher degradation of proteins is expected in lung diseases due to the release of proteases from inflammatory cells or/and from pathogens (e.g. *Pseudomonas aeruginosa*). Activities of several proteases, neutrophil elastase (NE), MMPs 1, 8, 9, and 12, caspases 3, 4, and 9, Cats D, E, G, K, and S, tryptases α and β , chymase, protease 3 and DPPIV were altered in COPD [37]. NE released from neutrophil granulocytes appears to be most important as it regulates CatB and MMP-2 of AMs, and

activates pro-MMP-2, MMP-7, and MMP-9 in various other cells [38]. Increased protease activities were observed for NE and MMP-9 in asthma, for NE, urokinase type plasminogen activator (uPA), MMP-1, MMP-8, MMP-9, MMP-2 in COPD, for NE, CatG, several MMPs, and proteinase 3 in CF, for CatB in lung fibrosis, and for MMP-2 and MMP-9 in pulmonary arterial hypertension (PAH). Based on these findings, it seems likely that degradation of biologics is increased in most lung diseases. Another problematic finding is the increase of inflammatory cells (mainly neutrophilic granulocytes and macrophages) in these diseases, which may lead to the generation of immune responses against the therapeutic protein.

Non-enzymatic barriers for biologics to reach target cells include increased thickness of the mucus layer in obstructive lung diseases. This may be combined with increased mucus viscosity and decreased mucus fibers mesh size, such as found in CF patients [39]. Coating of the protein drug with low molecular weight (up to 40 kDa) polyethylene glycol (PEG) may increase mucus penetration [3]. The increase of the air-blood barrier thickness caused by tissue remodeling in idiopathic pulmonary fibrosis (IPF) and other chronic lung diseases (e.g. COPD) is another barrier to delivery of biologics. The composition of the accumulated extracellular material is very complex and no strategies are available to improve permeation.

Reduced expression of tight junction proteins in respiratory epithelia of asthma and COPD patients and the disruption of the alveolar epithelium by migration of inflammatory cells in acute respiratory distress syndrome (ARDS) may theoretically increase drug absorption but the effect is too small to be relevant for most macromolecules [40].

3. Delivery of biologics by inhaler

The type of inhaler has critical importance for the delivery of biologics, also considering the fact that 75% of inhaled protein formulations in clinical research are produced as liquids [41]. Three classical types of inhalers are available for the delivery of orally inhaled products pressured metered dose inhalers (pMDI), dry powder inhalers (DPI), and nebulizers. Below, these three inhalers and soft mist inhalers (SMI) are described. pMDI, DPI and SMI are reviewed very briefly. Nebulizers as the most frequently used devices are described in more detail.

3.1. Pressured metered dose inhalers (pMDI)

The pMDI is a multi-dose, pressurized, aluminum canister that delivers drugs through a metering valve and orifice. pMDI contain drugs either as solutions or suspensions in a single propellant or propellant mixture. Patients inhale the aerosol in the plume produced by the device. Excipients such as ethanol or surfactants can be added to solubilize the drug or stabilize a drug suspension [42]. Excipients help

reduce crystal agglomeration, and coating the walls of the canister prevents the formulation from adhesion. Holding chambers and spacers decelerate the aerosol plume and reduce oropharyngeal deposition. Further details are omitted because biologics generally lack stability of the drug within the inhaler, absence of denaturation upon aerosolization and appropriate particle distribution.

3.2. Dry powder inhalers (DPIs)

DPIs either disperse individual doses pre-metered into capsules or blisters, or self-dose from bulk powder [43]. The active pharmaceutical ingredient (API) is provided either as pure drug particles or particles are blended to a carrier and drug particles are inhaled by the airflow generated by the patient. To facilitate de-agglomeration, various components inside the DPI, such as manifold, mesh, cyclone and spiral chamber are used. DPIs create a turbulent flow by increasing the resistance to airflow with the aim of increasing deposition. In general, high-resistance inhalers have higher deposition dose than low-resistance devices. A certain limitation of these devices is the high susceptibility of all DPIs to moisture. Most experience of delivering biologics with DPIs has been with Exubera® and Technosphere® insulin (Affreza®). However, spray-dried powder of infliximab could also be administered by DPI to mice in an asthma model [44]. To date, only three DPIs, the F1P for administration of the anti-IL-13 monoclonal antibody fragment VR942 (Abrezekimab), Cyclohaler® for administration of DAS181 (Fludase®) and Concept1 for administration of the anti-thymic stromal lymphopoietin (TSLP) monoclonal antibody Fab fragment (CSJ117) have been used in clinical trials. This indicates that proteins with the right formulation can successfully be administered to patients by dry powder inhaler.

3.3. Soft mist inhalers (SMIs)

SMI are novel multidose propellant-free handheld liquid inhalers and more suitable for delivery of biologics than pMDI. In all devices, an aqueous formulation is forced through nozzles but the methodologies to generate the droplets are different. Based on the definition of converting liquid into aerosol droplets, SMIs are similar to nebulizers. The use of SMIs for aerosolization of biologics is currently low. Biologically active IFN- ω and manganese superoxide dismutase could be delivered to >75% with Respimat® inhaler [45]. The AERx® system delivered IFN α -2b, DNase, and rh-IL-4 receptor more efficiently than jet nebulizer, demonstrating the suitability of SMIs for delivery of biologics [46-48]. Unfortunately, the AERx® system is no longer available. For the MedSpray® device, no data of biologics are available yet.

3.4. Nebulizers

Nebulizers are the most commonly used devices for pulmonary administration of biologics. Although nebulizers have specific disadvantages (e.g. long administration times, poor reproducibility, low delivery efficacy, high costs for maintenance, higher need of the expensive material, etc.), they have the advantage that they can be used for patients of all ages and of different disease stages. Three types of nebulizers are commercially available to generate 1–5 μm droplets (Fig. 3). As not all nebulisers are equally suitable for protein delivery, the underlying technology is explained. Nebulizers have the problem that the large air-liquid interface (24m² to 1500 m²) interferes with protein delivery and causes denaturation in a multi-step process [49]. The proteins are absorbed at the interface and the molecules align in a flat conformation. This includes partial unfolding and exposure of hydrophobic parts normally hidden in the protein structure. The exposure is associated with an increase in surface activity and decrease of biological activity. After this rapid step, further changes based on the secondary structure of the protein and layer of adsorption occur. These include aggregation in the air-liquid interface and around a non-native protein desorbed from the interface in the bulk solution. Partial unfolding also affects thermal degradation, leading to lower melting temperatures of the molecules. The major contribution of the air-liquid interface to degradation has been demonstrated when a jet nebulizer was immersed in protein solution and no interaction with the air-liquid interface could take place [50]. In this setting, nebulized G-CSF was not degraded. Recirculation is thought to be a more important factor for protein denaturation than shear forces. When operating air pressure was increased as the main factor for shear forces, no change in the degradation of urease was observed, provided aerosol recirculation was excluded [51].

3.4.1. Jet nebulizers

Jet nebulizers work with compressed gas that passes through a narrow orifice, causing a region of negative pressure. A gas stream entrains the solution or suspension and shears it into a liquid film. Due to surface tension, the film breaks into primary aerosol droplets with broad distribution between <1 to 600 μm . An array of baffles filters droplets of inadequate size and larger droplets are recycled into the fluid reservoir. The respirable fraction of the generated aerosol is in the range of 30-70% of the fill [52]. The most common jet nebulizers with a corrugated tube deliver the aerosol continuously during inhalation and exhalation phases, which leads to waste of medication and only 5-15% of the initial charge deposits in the lungs. Aerosol waste during expiratory phase can be reduced by using jet nebulizers with a collection bag (e.g. Circulaire®) and breath-enhanced dosimetric design [53]. Breath-enhanced nebulizers (BEN) release more aerosol during inhalation through one-way valves in the mouthpiece. Common BENs are PARI LC Plus®, PARI LC®-D, PARI LC Sprint®, SideStream Plus®, and

NebuTech®. Breath-actuated nebulizers (BANs) like AeroEclipse® II sense the patient's inspiratory flow and deliver aerosol only on inspiration.

3.4.2. Ultrasonic nebulizers

Ultrasonic nebulizers convert electrical energy to generate high-frequency ultrasonic waves [54]. A piezo-electric crystal oscillation at frequencies >1MHz is embedded in the bottom of the instrument. The waves are transferred to the surface of a liquid and an aerosol is produced. The exact mechanism is not clear: either capillary waves formed on the liquid surface or cavitation by collapsing microscopic air bubbles create the aerosol droplets. Higher energy input is correlated to smaller droplet size. The resulting aerosol is polydisperse and an internal baffling system is used to obtain droplets in the desired size range. Ultrasound nebulizers are more portable and operate quietly, but drug inactivation is more frequent with this type of nebulizer than with the other types. This is because, in addition to interacting with the air-liquid interface, ultrasound and cavitation can damage proteins. Another limitation is that ultrasonic nebulizers are not able to aerosolize viscous solutions. Furthermore, they dissipate a large part of the introduced energy as heat, resulting in temperatures in the reservoir up to 75°C.

3.4.3. Vibrating mesh nebulizers

Vibrating mesh nebulizers use a vibrating mesh or plate with multiple apertures to produce the aerosol [53]. This may be a piezoelectric actuator coupled to a mesh with a horn vibrating ultrasonically against a static mesh, so called passive, realized in MicroAIR® NE-U22 and similar models and I-neb®, or actively with a mesh mounted in an ultrasonically vibrating piezo ring (PARI eFlow®, Aeroneb® Pro and Aeroneb® Go). Advantages of vibrating mesh nebulizers are that no baffling system and no aerosol recirculation is needed to generate primary aerosols with a narrow size distribution. The technology leads to lower concentration of the solution in the reservoir and is associated with low shear stress. The air-liquid interface is reduced compared to jet nebulizers and only a small increase in temperature for the NanoSpray Dryer B90 was observed. Vibrating mesh nebulizers reduced the time to deliver the aerosol 3-4 fold and increased lung deposition 2-4 fold compared to jet nebulizers. BANs for vibrating mesh nebulizers are microprocessor-based (I-neb AAD® system) [55]. The AKITA system can be combined with a jet (AKITA® JET) or mesh nebulizer (AKITA® APIXNEB). In combination with a mesh nebulizer lung deposition is ~70% of the nominal nebulizer fill. Adaptive aerosol delivery (AAD®) systems, like Prodose AAD® and AKITA®, deliver the drug dependent on the patient's breathing and the type of drug. Aerogen-Pro® and Aerogen-Solo® are BAN that can be used in non-invasive and invasive nebulization [56], making them particularly suitable for geriatric patients, children and critically ill patients. For mechanical inhalation, devices with an additional filter on the exhalation port must be used to prevent nosocomial infections (e.g. COVID-19 patients). The I-neb AAD™ system efficiently delivers inhaled alpha 1 proteinase inhibitor (previously termed alpha-1 antitrypsin; a1-PI)

for treatment of CF to protect the lungs from excessive free elastase [57]. AKITA² APIXNEB[®] system has been used for pulmonary delivery of recombinant granulocyte macrophage colony stimulating factor (rGM-CSF), Dornase alfa and Nanobody[®] ALX-0171 [20].

The FOX[®] vibrating mesh nebulizer is a small, handheld, breath-activated, battery-powered inhalation system suitable for the delivery of small molecules and biologics, formulated as solutions or nano-suspensions. More recently developed vibrating mesh nebulizers have more holes (1000 – 15,600) for smaller droplet size and faster flow rates [58]. The examples for mesh nebulizers given here are not exhaustive and the reader is referred to reviews dealing with nebulizers (e.g. [3, 56, 58]).

More recently developed Surface Acoustic Wave (SAW) nebulizers generate short-frequency sound waves that travel along the surface of a liquid to create the aerosols. They have similar advantages to vibrating mesh nebulizers, but in addition cause less clogging, making them particularly suitable for the nebulization of monoclonal antibodies and peptides [58]. Hybrid resonant acoustic nebulizers (HYDRA) improve on the low nebulization rate of SAW nebulizers and also avoid shear stress. However, only small molecule data are currently available [3].

3.4.4. Comparison in the performance for delivery of biologics

The comparison between nebulizers and SMIs highlights the specific advantages and disadvantages of each aerosolizers in terms of key performance parameters for biologics: Precise and reproducible dosing, delivery of high doses, minimal drug loss and good deposition. [41]. There is no optimal device for all parameters, in general, SMIs and SAWs are optimal in terms of protein integrity and precise dosing, while all conventional nebulizers deliver higher doses.

Significant multimerisation and aggregation of Nanobody[®] ALX-0171 was observed when aerolised with jet nebulizer, while the effects was minimal after nebulization with vibrating mesh nebulizer [59]. Studies assessed the preservation of activity/structural integrity of a range of peptides and proteins and reported that stability of peptides against damage by nebulization varies and is not linked to molecular weight. a1-PI (41/51kDa), secretory leukoprotease inhibitor (12kDa), DNase (30kDa), heparin (3-20kDa), GM-CSF (14.5kDa), insulin (12-20kDa), Il-1 receptor antagonist (17.2 kDa), tissue plasminogen activator (t-PA, 6kDa) were stable, while G-CSF (18.8kDa), pegylated recombinant G-CSF (24-36kDa), IFN- α (19kDa) and growth hormone (22kDa) were unstable [60]. For the biologics delivery, the vibrating mesh technology provided the highest activity (90-100%) of the DNase product Pulmozyme[®], followed by jet nebulization (80-96%) and ultrasound technology (40%) [61, 62]. a1-PI activity was preserved to 90-95% with PARI eFlow[®] nebulization [63], while information on activity loss after nebulization with jet technology was not available [64]. In a comparison between the three nebulizer types, activity of Erbitux[®] (cetuximab) was best maintained with vibrating mesh technology

[65]. Aggregate formation of insulin growth factor (IGF)-I after jet nebulizer aerosolization by PARI LC® Sprint and vibrating mesh nebulizer aerosolization with PARI eFlow® was similar [66]. The output rate with PARI eFlow® was four times higher, but the fine particle fraction (FPF) was lower than with the jet nebulizer.

4. Strategies for delivery of biologics to the lungs

Most of the inhaled biologics are liquid suspensions of the proteins/peptides without modifications, and delivery is mainly determined by aerosol droplets deposition. However, there are opportunities to improve pulmonary delivery through modifications that target specific uptake routes, increase mucus permeation, prevention degradation or improve resistance to aerosolization [20].

4.1. Targeted permeation

In fusion proteins, the protein of interest is coupled to the Fc fragment of antibodies. The preferential expression of the FcRn in the upper airways will result in an increased delivery in this area. Erythropoietin (EPO), IFN- β and follicle stimulating hormone (FSH) were delivered in this way to the lungs in preclinical studies [21, 67, 68].

4.2. Increase of mucus permeation and prevention of degradation

The most important factors influencing the interaction of small peptides with mucus are size and polyvalency [69]. Reduction of molecule size is the preferred strategy for pulmonary delivery of antibodies. Anti-IL-13 Fab fragments, anti-tumor necrosis factor (TNF)- α single domain antibodies, and nanobodies are promising biologics that will be discussed in section 4.1.

Engineering the charge and pegylation are applied strategies to regulate the mucus binding. While mucus permeation enhancing effects of PEG have been mainly reported for nanocarriers, few data are available from pegylated proteins and peptides. Pegylation of the cationic antimicrobial protein lysozyme reduced the interaction with the polyanions in CF lung mucus and improved antimicrobial activity [69]. Another effect of PEG is its effect on protein stability by preventing degradation.

Generally, conjugation can be performed with a series of carbohydrate moieties or synthetic polymers, e.g. glycans, PEG, dendrimers, polyethylenimine (PEI), hyaluronic acid, etc. [70]. PEG is a biocompatible, neutral, linear polyether available in a wide range of molecular weights. The chemical structure of this molecule provides both hydrophobicity and affinity to water, due to the existing repeated ethylene moieties and the oxygen in between. Oxygen provides strong interactions with water and a high level of hydration [71]. The large flexibility and hydration properties of PEG make it sterically stable and the steric hindrance prevents access of proteases, binding of antibodies and opsonins [72, 73]. However, a fine balance is required to avoid the interference of the PEG chain with

the biological activity of the drug [74]. Anti-IL-17A F(ab')₂ and anti-IL-13 Fab' were conjugated to a two-armed 40-kDa PEG chain to prolong their residence time in murine lungs. The content of PEGylated antibody fragments within the lung increased up to 4h after lung delivery, whereas the clearance of unconjugated proteins started immediately after administration [75]. Pegylation has also been used to increase the retention of IFN- α [76]. Degradation can also be reduced by chemical modification, which has been used for the plant derived actin-inactivation resistant (AIR) DNase to prevent actin-inactivation of recombinant human DNase in CF mucus [77].

Besides PEGylation of the biologic, it is also possible to PEGylate the carrier. PEGylated liposomes are provided by using a mixture of phospholipids as the main component with PEGylated phospholipids for the delivery of small molecules via inhalation [78, 79]. It was reported that using PEG chains with a molecular weight between 1000 – 2000 Da and a density of 5–10 mol% of the total lipid, provides the optimum characteristic for drug delivery [80]. Patel et al. [81] used Large porous hollow particles (LPHPs) and PEGylation for encapsulation of low molecular weight heparin in PEG-PLGA large porous particles, for delivery to the lung. They reported uniform distribution of particles in the lungs. Further, compared to not pegylated PLGA particles, reduced uptake by AMs was noted [82, 83].

4.3. Stabilization of biologics

The use of excipients to stabilise formulations for pulmonary delivery is more difficult than for oral products because only a limited range of substances is approved for this route of administration. Mainly lipids, especially 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), amino acids and sugars, which can be used because they are recognized as Generally Recognized As Safe (GRAS) excipients for pulmonary drug delivery by the Food and Drug Administration Agency (FDA).

4.3.1. Modification and drying

Stabilization against shear stress, cavitation and oxidative stress by nebulizers can be prevented by chemical modification of the proteins. The replacement of free cysteine with serine residues was reported to hinder the formation of intermolecular disulfide bonds and stabilizes IFN- β and IL-2 [84].

A more universal option would be to dry the proteins, as degradation is water-mediated and biologics are mainly provided as liquid formulations [85]. Spray-drying is the preferred method and stabilizing agents such as sugars and polyols, salts, surfactants and amino acids, help to protect the conformational structure of the biologic. The most commonly used sugars can decelerate degradation pathways of the biologic molecule in the solid state, by trapping the molecule in a rigid and amorphous glass matrix. This reduces local and global mobility, through interactions such as hydrogen bonding [86]. Surfactants, like polysorbate 80 and DPPC, can be also used to prevent aggregation by avoiding adsorption of the biologics at the air-liquid interface or by hindering ice-liquid interfaces during the

drying process [87, 88]. Further, amino acids such as arginine, histidine and glycine and their combinations have been used as stabilizers during drying of proteins [89]. As an example, Spray freeze-dried adalimumab microparticles for inhalation were stabilized using leucine or phenylalanine [90]. Combinations of salts, sugars and amino acids as stabilizers are used in dry powders of Fab fragments VR942 and CSJ114 (section 4.1).

4.1.2. Nano-encapsulation of biologics

Different techniques have been used for encapsulation of insulin, while less data are available for other biologics [91]. Liposomes are the most commonly used and most advanced formulations in clinical development for pulmonary delivery, but their efficacy for biologics appears to be limited [92]. Liposomal uptake of leuprolide to increase serum luteinizing hormone showed low (59%) bioavailability compared to subcutaneous administration [93]. Pulmonary administration of liposomal IL-2 in phase I to lung metastases of various carcinoma was not toxic, but also not efficient [94]. Similarly, a phase I trial of liposomal IL-2 in patients with various immunodeficiencies was not effective [95]. Shear during the nebulization may exert physical stress on liposome bilayers, resulting in losses of the originally entrapped drug. In addition, the slow release from the carrier may allow for higher protein degradation. It was shown that degradation of liposome-encapsulated calcitonin was higher after intratracheal aerolization in male Wistar rats compared to an i.v. injection [96].

Erythropoietin (EPO), extendin-4, and calcitonin could be incorporated into polylactic acid/polylactic-coglycolic acid (PLA/PLGA)-based particles [90]. However, their stability and biological activity remain unclear as several peptides, namely calcitonin, octreotide, goserelin and leupeptide, were acetylated by interaction with PLGA. On the other hand, the particles, might allow selective targeting of AMs. Ruge et al. reported that coating with mannose and PEG resulted in increased uptake by macrophages *in vitro* and *in vivo* via binding of mannose to surfactant protein A [97]. The mucolytic α -chymotrypsin can be encapsulated in novel biodegradable PEG-co-polyester microparticles and delivered as dry powder with minimal loss in activity [98].

No biologics have yet been delivered using nanocarriers. However, if carriers are used, strategies have to be found to prevent uptake by AMs [3] and approved excipients should be used for engineering the encapsulation. To prevent the AM uptake, coating with large hydrophilic polymers (e.g. PEG) may be an option.

5. Biologics in clinical trials for treatment of lung diseases

Compared to insulin, there is relatively little data available for oral inhalation of other biologics. Therefore, the most promising candidates will be presented individually, ordered by their effect as

antibodies, cytokines, enzymes and other proteins. A summary of published and active clinical trials with inhaled biologics can be found in Table 1.

5.1. Antibodies and protein scaffolds

Use of F(ab)₂, fragments, Fab fragments, Fc fragments, single chain fragment variable region (scFvs) and single variable domain heavy chain antibodies (sdAb) have been developed to improve tissue penetration and decrease uptake by phagocytes [99]. High-affinity anti-human IL-13 monoclonal Fab fragment VR942 blocks the pro-inflammatory action of IL-13. VR942 is a spray-dried powder containing leucine, trehalose and buffer salts as stabilizers used in combination with Vectura dry powder inhaler (<https://patents.google.com/patent/WO2018078186A1/en>). VR942 was tested in asthma patients in phase I trials and reported to be safe [100]. Clinical efficacy was shown by statistically significant rapid and durable reduction of exhaled FeNO concentration

CSJ117 is an anti-thymic stromal lymphopoietin (TSLP) monoclonal antibody Fab fragment. TSLP is a type I cytokine, belonging to the IL-2 family that promotes and amplifies Th2 immunity and, this shifts the immune response from tolerance to pro-inflammatory response [101]. The Fab fragment is supplied as spray-dried formulation stabilized with leucine, trileucine, mannitol, and trehalose and is administered using the Concept1 device, which is a single dose DPI. CSJ117 has been tested in a phase I trial and was reported to be safe and well tolerated in adult mild asthmatics. Furthermore, it reduced allergen-induced bronchospasm [102]. It is now being evaluated in a phase II trial for use in asthma, which could not adequately be controlled with medium to high dose inhaled corticoids and long acting beta antagonists.

Single domain antibodies represent the smallest functional antigen-binding units of antibodies (10–13 kDa). The variable parts of human heavy chains require coverage of the hydrophobic parts at their surface by light chains, to be stable in physiologic solutions. The structure of camelid (camel and lama) antibodies is different and single domain heavy chain antibodies, when prepared in the correct manner, do not aggregate [103]. Such antibodies (nanobodies) are sufficiently stable to allow nebulisation [104]. The nanobody ALX-0171 (Gontivimab) was designed to target the respiratory syncytial virus (RSV) surface fusion protein and applied by FOX-Flamingo® inhalation system. One phase II clinical trial showed dose-dependent anti-viral effects, while another study reported lack of clinical efficacy [105]. A follow-up phase II has been withdrawn and further development of ALX-0171 was stopped by the producing company (Ablynx) [106]. Nanobody Nb 11-59 against the SARS-CoV-2 spike glycoprotein successfully blocked virus entry *in vitro* and remained stable upon nebulization [107]. The alpaca-derived single domain antibody fragment Ty1, fused to Fc fragment, is another promising candidate for treatment of COVID-19 patients using this technology [108].

GSK 1995057 is a fully human, single domain anti-TNF receptor 1 antibody. The antibody applied by PARI eFLOW® nebulizer was tested in healthy volunteers challenged with low endotoxin and reduced neutrophilic granulocytes and cytokine release, in BALF and serum. Furthermore, decreased endothelium injury was demonstrated in the phase I trial. Despite these promising results, the company stopped further development of the product, because naturally occurring pre-existing anti-drug antibodies were detected [109]. In the improved product GSK 2862277, a C-terminal alanine residue was added, to render the product less susceptible to pre-existing antibodies. This antibody, administered by PARI eFLOW® nebulizer, has successfully been tested in a phase I clinical trial, but the phase II trial evaluating the action on acute lung injury was discontinued [110].

As alternatives to antibodies, over 50 different protein scaffolds, termed domain-sized compounds and constrained peptides, have been developed. These synthetic binding proteins are produced by altering parts of functionally inert proteins, which act as a scaffold [111]. Protein scaffolds relevant for inhalation are Anticalin® proteins. They have a molecular weight of 6-20 kDa and are generated from natural lipocalins, which are present in human plasma [112]. Lipocalins transport small molecules (e.g. retinol binding protein for retinol or apolipoprotein D for progesterone and arachnidonic acid) or have a scavenger function (e.g. neutrophil gelatinase-associated lipocalin that binds bacterial siderophores and thereby restricts their iron supply). There is some similarity between anticalin proteins and Fab fragments because both have variable loops in the binding sites; these are four for anticalins and six for Ig. By reshaping the binding pockets of natural lipocalins, novel binding functions are created and anticalin proteins are formed. IL-4 receptor α , targeting anticalin protein PRS-060 (AZD1402) applied by InnoSpire Go mesh nebuliser for asthma, according to an interim report of an ongoing phase I trial reduced fractural exhaled nitric oxide (a valid biomarker for eosinophilic airway inflammation). The reduction was 36% relative to placebo-treated patients [113]. The protein is also available as a dry powder for administration with Plastiaple Monodose inhaler.

5.2. Cytokines

Application of IFN- α by I-neb® nebulizer to patients with IPF was safe and lung function remained stable over the 80 weeks of the study [114]. IFN- β 1a (SNG001, Synairgen) has been tested for safety in asthma patients using a vibrating mesh nebulizer. Anti-viral responses were obtained according to sputum biomarkers and, based on these results, the protocol of an on-going phase II trial with COPD patients has been adapted to include patients with confirmed SARS-CoV-2 infection and pre-existing impairment of lung function [115]. Phase II trials with IFN- γ in CF with lung infections have been initiated, where IFN- γ was administered as Actimmune® with jet nebulizer. Application was well tolerated but no benefit was observed in terms of bacterial load, inflammation in sputum and lung function. The drug has been also tested in IPF, where it was safe and improved oxygen diffusion

capacity [116]. Vibrating mesh nebulizers Aerogen® Solo and photo-defined aperture plate PDAP® were able to deliver biologically active IFN- γ in droplet size, that may reach the deep airways and may improve therapeutic efficacy [1].

A phase I trial studying the action of nebulized recombinant IL-2 (aldesleukin) for metastatic or unresectable cancer was terminated without available results. However, in 2003, orphan designation (EU/3/03/146) was granted by the European Commission for inhaled aldesleukin for the treatment of renal cell carcinoma. The treatment was safe and effective, but temporarily caused asthma-like inflammation [117]. This side effect is not surprising as IL-2-dependent resident Th2 memory cells are drivers of lung allergic responses. The recombinant IL-4 variant AER-001 (pitrakinra) is known as a “mutein”, which is a mutant of a normal agonist. It binds to the α -subunit of the IL-4 receptor and prevents inflammation induced by IL-4 and IL-13. The protein was delivered as spray-dried sucrose- and citrate-stabilized formulation AEROVANT™ AER001 (<https://patents.google.com/patent/WO2011156000A2/en>) and by PARI LC Plus nebulizer to asthma patients in a phase II trial. The observed effects were small. Only in a subgroup of patients with eosinophilic asthma the rate of exacerbations fell by 37%, where pitrakinra was used as dry powder. When delivered by nebulizer, the decrease in forced expiratory volume in 1 second (FEV1) after challenge with methacholine, was less pronounced compared to placebo [118].

Sargramostatin (Leukine®) and molgramostim (Molgradex) are human recombinant GM-CSF and were tested in phase II trials for autoimmune pulmonary alveolar proteinosis (aPAP). This is a rare disease, where neutralising GM-CSF antibodies and alveolar surfactant accumulation impair lung function. A study on 64 patients randomly assigned to sargramostin or placebo inhalation showed that the alveolar:arterial oxygen gradient was improved with GM-CSF, but effects were very modest [119]. The combination of whole lung lavage and inhaled sargramostin aerosolized by PARI BOY® jet nebulizer was well tolerated and more efficient than lavage alone [120]. Sargramostatin has been tested in COVID-19 patients and improved lung function measured by oxygenation levels and markers for disease severity and lung fibrosis and induced specific immune responses (<https://www.prnewswire.com/news-releases/sarpac-clinical-trial-of-leukine-sargramostim-rhu-gm-csf-in-hospitalized-covid-19-patients-meets-primary-endpoint-of-significant-improvement-in-lung-function-301236275.html>), according to the company's press release. It should be mentioned that according to the study protocol nebulized sargramostatin was replaced by intravenous administration after 5 days. As the publication of the results is not yet available, it is unclear whether the improvement is due to nebulization or to intravenous injection of sargramostatin. Molgradex aerosolized by vibrating mesh nebulizer PARI eFlow®, improved oxygenation status and clinical symptoms. However, the phase III trial did not meet the primary endpoint because the difference in the alveolar:arterial oxygen

gradient between molgradex and the placebo group was only 3.3 mm Hg. Despite the suboptimal result, FDA has granted Molgradex orphan drug designation for autoimmune PAP. Molgramostim was further being tested against nontuberculous mycobacterial infection in CF patients. However, due to recruitment and study conduct issues, all related to the COVID-19 pandemic, this trial was stopped (<https://www.oindpnews.com/2020/09/savara-discontinues-development-of-molgradex-for-ntm-lung-infections/>). A phase II study currently recruiting will evaluate the efficacy of Molgradex to prevent ARDS in COVID-19 pneumonia.

5.3. Enzymes

Dornase alpha (Pulmozyme®) containing recombinant human deoxyribonuclease I is approved by the FDA for treatment of CF. It removes extracellular DNA and actin accumulated by death of neutrophilic granulocytes, and improves mucus viscosity in CF and COPD. Pulmozyme® is now also under evaluation for other applications, namely respiratory failure (phase III) and neutrophilic asthma (phase II) trials. Dornase alpha recently also has been administered to a mechanically ventilated individual with severe COVID-19 infection. The beneficial action on the disease was explained by cleavage of the sticky large chromosomal DNA from neutrophil extracellular traps produced by infiltrating neutrophils [121]. Since Dornase alpha activity is inhibited by actin that accumulates in the sputum of CF patients, a chemically modified plant cell-derived DNase 1 was developed. A phase II switchover trial in CF patients showed that AIR DNase™ (alidornase alpha) was well tolerated in the patients [77]. AIR DNase™ is applied by vibrating mesh nebulizer.

Recombinant neuraminidase (DAS181, Fludase®) is a novel sialidase fusion protein provided as a dry powder stabilised with histidine, trehalose, and electrolytes [122]. DAS181 for indication in influenza and parainfluenza infections administered with Cyclohaler® and aerosolized with nebulizer was found to be safe in healthy individuals and mildly asthmatic patients according to phase II trials [123]. A phase III trial has been recently started. DAS181, containing the catalytic domain of *Actinomyces viscosus* sialidase, removes cell surface sialic acid and, by removal of the attachment sites, prevents attachment of influenza and parainfluenza virus to cells [124]. The concern that removal of sialic acid residues may increase colonization with *Streptococcus* sp. was not confirmed, as the rate of secondary infections actually decreased. After having shown efficacy in a previous clinical trial for compassionate use in four COVID-19 patients, the company was able to obtain an add-on to the running phase III trial to evaluate DAS181 in severely ill COVID-19 patients [125]. While NCT04354389 with a planned start in May 2020 has been withdrawn, two other trials (phase II and phase III) are active and will assess efficacy of DAS181 in COVID-19 patients.

Tissue plasminogen activator (alteplase) is a serine protease (EC 3.4. 21.68) involved in the breakdown of blood clots in plastic bronchitis, which is a rare complication of respiratory diseases and secondary

phenomenon after Fonton surgery, in patients with congenital heart disease. The application of the recombinant enzyme by Pari LC sprint® nebulizer was successful in case studies (e.g. [126]) and is now tested in phase II trials.

5.4. Other proteins

Altrakincept is a recombinant, human, soluble IL4R α protein that binds to IL4 and competitively inhibits its binding to IL4 receptors on cell surfaces. In patients with moderate asthma, lung function was improved and airway inflammation decreased after application of nebulised altrakincept once a week [118]. It was suspected that the failure of the drug in a follow up phase III trial was due to insufficient bioavailability, caused by high proteolytic enzyme levels in the lungs of asthma patients [127].

a1-PI applied by vibrating mesh nebulizer was tested in phase III trials in patients with a1-PI deficiency. The glycoprotein represents a physiological inhibitor of the serine proteases trypsin and chymotrypsin. It also inhibits elastase produced by neutrophilic granulocytes in the lungs. Upon a1-PI deficiency, elastase is not sufficiently inhibited and increased degradation of elastin and collagen leads to emphysema. Treatment of CF patients with a1-PI nebulized by AKITA² APIXNEB® nebulizer system was well tolerated [128]. The inhibitor aerosolized by PARI eFLOW® nebulizer was also safe and efficient in another phase II trial with CF patients. It reduced airway inflammation and *Pseudomonas aeruginosa* in sputum, while lung function was not affected [64]. The insufficient effect on lung function may be explained by the fact that other proteases, which play also a role in CF, were not inhibited (e.g. cathepsins, metalloproteases) [129]. The limited effect in the phase II trials with CF patients does not indicate that a1-PI could not be active in patients with a1-PI deficiency, where mucus viscosity is lower and penetration of the protein therefore better.

Solnatide (AP301) is a synthetic peptide of around 20 aa and an activator of the sodium ion channel (ENaC) of alveolar epithelial cells. After successful phase I safety study, the peptide was applied in a phase II study as aerosol to mechanically ventilated patients with ARDS hospitalized in intensive care units. Solnatide reduced pulmonary permeability oedema and moderate-to-severe ARDS [130]. Based on these results, a clinical trial for compassionate use in COVID-19 patients with ARDS or pulmonary edema has been granted [131].

Coagulation plays a role in ARDS pathophysiology and heparin has anticoagulant and anti-inflammatory properties [132]. Various clinical trials were performed with nebulized heparin in combination with N-acetylcysteine and bronchodilator in patients with burn trauma, but also alone in phase I trials and for patients at risk for ARDS. Results on efficacy were contradictory, which may be due to the use of different types of nebulizers, dosages and disease severity [133]. Currently, nebulized unfractionated heparin is being tested in phase III trials for ARDS. A phase II/III trial, which is not yet

recruiting, will evaluate the beneficial effect of nebulized unfractionated heparin in severely ill COVID-19 patients.

ALX-009 is a combination of two substances found naturally in the body, hypothiocyanite (OSCN-) and lactoferrin. Both substances are deficient in CF patients and substitution is expected to increase resistance against bacterial infections [134]. Administered by nebulizer, ALX-009 is being evaluated in phase I in CF and bronchiectasis patients.

Myristoylated alanine-rich C-kinase substrate (MARCKS) is the central regulatory protein linking secretagogue stimulation of goblet cells and BIO-11006 is 10aa synthetic peptide that inhibits its function. According to results from the safety study, the peptide reduced mucus hypersecretion in airway cells of COPD patients and improved their lung function [135]. It is tested for ARDS, non-small cell lung cancer and lung metastases of sarcoma in phase II trials as nebulized aerosol. Further, administered twice daily, BIO-11006 showed beneficial effects in COPD patients by increasing FEV1 [136].

POL 6014 neutrophil elastase inhibitor (Lonodelestat) for inhalation with a PariFlow® system has passed a safety study in healthy volunteers and patients with CF [137]. Beneficial effects may be expected because activity of elastase in sputum of CF patients was decreased. The company, Synthera Pharmaceuticals, collaborates with Cold Spring Harbor Laboratory to investigate Lonodelestat also for ARDS in patients with COVID-19 infections [138].

With the exception of Alidornase, no chemically modified proteins or encapsulated biologics are used. Administration is mainly in liquid form by nebuliser, fewer molecules can be applied both in liquid form and as dry powder, and few are available only as dry powder. With regard to the efficacy of biologics, it should be noted that mainly results from phase I and II trials are available, in which no conclusive assessment on efficacy is possible. It is apparent that the development of some products has been discontinued despite promising effects in these phases (e.g. ALX-0171, GSK 2862277, and altrakincept). For other biologics, such as pitrakinra, IFN- γ , sargramostim, and α 1-PI, only small effects are seen in some indications. While VR942, CSJ117, and IFN- β 1a are still being tested in phase II trials, AIR DNase, α 1-PI, DAS181 and heparin are being tested in phase III trials.

To identify biologics that may enter clinical trials in the near future, results from pre-clinical studies published in the last 10 years are listed in Table 2. Encouraging studies published before this time were either not followed up or could not be confirmed in later studies. For example, inhalation of fusion proteins of Fc fragments with EPO, IFN- β or FSH were published almost 20 years ago, but despite promising results in a phase I trial, it is currently not being investigated further [20]. The situation is similar with the administration of human growth hormone.

Table 2 shows that various proteins and peptides are being investigated for lung diseases. Studies have mainly investigated lactoferrin, vasoactive intestinal peptide derivate, and antibacterial peptides. In terms of formulation, similar to more advanced biologics, mainly the unmodified proteins have been administered.

6. Risk by inhaled biologics

In contrast to the gastrointestinal tract, the capacity of the respiratory tract to degrade and remove foreign material is low. The lungs possess two major clearance mechanisms, mucociliary clearance and phagocytosis by alveolar macrophages and accumulation of inhaled biologics may cause overload of these mechanisms. Phospholipidosis of macrophages may be an indication for impaired function of the clearance system of the respiratory part of the lung [33]. Another potential risk may be generation of immune reactions triggered by denaturated or chemically modified proteins that are recognized as “foreign” by the immune system.

7. Conclusion

Degradation by proteases, shear stress, and induction of immune responses by denaturated proteins and aggregates complicate pulmonary delivery of biologics. Protein susceptibility varies and currently no strategies proved to be suitable for all proteins. Pegylation may improve mucus penetration and stability against proteases, while antibody fragment generation has been successful for antibody treatment. In contrast to small molecules, encapsulation in carriers did not lead to biologics in clinical trials. This could be due to lack of stability for liposomes and interactions of the biologics with the carrier material in PLA/PLGA particles. The limited number of approved excipients for safe delivery to the lung is the main obstacle for particle engineering and development of effective and advanced encapsulation strategies. While development of several biologics for pulmonary administration have been halted or had little positive impact, the antibody fragments VR942 and CSJ117, and IFN- β 1a are being investigated in phase II trials. Due to the urgent need for effective treatment of COVID-19 patients, a large number of inhaled biologics are being evaluated in clinical trials for this indication. These biologics include nanobody Nb11-59, IFN- β 1a (SNG001), molgramostim (Molgradex), sargramostim (Leukine®), dornase alpha (Pulmozyme®), DAS181 (Fludase®), heparin, POL6014 (lonodelestat), and AP301 (solnatide). A phase IV trial with inhaled and parenteral sargramostim (Leukine®) reported improvement of lung function and specific immune responses. It is expected that the increasing number of trials for the treatment of COVID-19 will lead to improvements in pulmonary administration of biologics.

Declarations of interest

None

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Fig. 1. Specific challenges in the administration of biologics by inhalation showing influencing conditions (violet arrows), consequences (red arrows), and measures for improvement (green arrows).

Abbreviations: CF, cystic fibrosis, COPD, chronic obstructive pulmonary disease; IPF, idiopathic pulmonary fibrosis.

Fig. 2. Architecture of the respiratory system with scheme of bronchiole as the last part of the conducting airways and alveole as representative of the respiratory part. In the bronchioles, bronchial epithelial cells (BC) and goblet cells (GC) from the epithelial layer. They are covered with mucus (M). In the lamina propria dendritic cells (DC), smooth muscle cells (SC), lymphocytes (L) and fibroblasts (F) are found. The surface of the alveoles is lined by alveolar epithelial cells (AC) with are covered by surfactant (S). Alveolar macrophages (AM) patrol over the surface in the surfactant layer. Below the alveolar epithelium endothelial cells (EC) are located. Abbreviations: B, blood.

Fig. 3. Operation scheme of the three common types of nebulizers. Jet nebulizers deliver compressed gas through a jet, which causes a region of negative pressure. The solution or suspension to be aerosolized is entrained into the gas stream and is sheared into a liquid film. This film is unstable and breaks into droplets because of surface tension forces. A baffle in the aerosol stream produces smaller particles. In ultrasonic nebulizers an alternating electric field is applied to a piezoelectric transducer, which converts the electrical signal into a periodic mechanical vibration. The vibrations are transmitted through a buffer to the drug solution and form a fountain of liquid in the nebulization chamber. A baffle is used to reduce droplet size of the aerosol. In vibrating mesh nebulizers contraction and expansion of a vibrational element produces an upward and downward movement of the aperture plate. The holes of the mesh have a tapered shape with a larger cross-section on the liquid side and a smaller cross-section on the side the droplets emerge. Aerosol particle size and flow are determined by the exit diameter of the aperture holes.

Table 1. Overview of clinical trials with inhaled biologics (A, active; I, enrollment by invitation; NR, not yet recruiting; R, recruiting)

Type of biologic	NCT Number	Title	Status	Condition(s)	Inter
Antibodies	NCT03574805	Study of Multiple Doses of PRS-060 Administered by Oral Inhalation in Subjects With Mild Asthma	R	Asthma	Drug Place
	NCT03574805	Study of Multiple Doses of PRS-060 Administered by Oral Inhalation in Subjects With Mild Asthma	R	Asthma	Drug Place

	NCT04410523	Study of Efficacy and Safety of CSJ117 in Patients With Severe Uncontrolled Asthma	R	Asthma	Drug Drug
Cytokines	NCT04569877	GM-CSF Inhalation to Prevent ARDS in COVID-19 Pneumonia	R	Severe Acute Respiratory Syndrome (SARS) Pneumonia COVID-19 Pneumonia	Drug nebu Other nebu
	NCT03421743	Pilot Trial of Inhaled Molgramostim in Nontuberculous Mycobacterial (NTM) Infection	A	Mycobacterium Infections, Nontuberculous	Drug molg
	NCT03482752	Safety Extension Trial of Inhaled Molgramostim in Autoimmune Pulmonary Alveolar Proteinosis	I	Autoimmune Pulmonary Alveolar Proteinosis	Drug
	NCT02595060	Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF) Inhalation to Improve Host Defense and Pulmonary Barrier Restoration	R	ARDS	Drug molg (rhG inhal
	NCT04707664	Sargramostim Use in COVID-19 to Recover Patient Health	NR	Covid19 SARS-CoV Infection	Sargr Place
	NCT04642950	A Phase II/III Study of Sargramostim	R	COVID-19	Sargr Place
	NCT04569877	GM-CSF Inhalation to Prevent ARDS in COVID-19 Pneumonia	R	Severe Acute Respiratory Syndrome (SARS) Pneumonia COVID-19 Pneumonia	Molg nebu solut Place solut
	NCT04411680	Study of Sargramostim in Patients With COVID-19	A	COVID-19 Sars-CoV2	Sargr Stand
	NCT03570359	A Study to Test a Potential New Treatment for COPD Patients Suffering From the Common Cold or Influenza	R	Chronic Obstructive Pulmonary Disease (COPD)	Drug Beta- Place
Enzymes	NCT03994380	Use of DNase in Neutrophilic Asthma	A	Neutrophilic Asthma	Drug Inhal
	NCT03368092	Inhaled Dornase Alpha to Reduce Respiratory Failure After Severe Trauma	R	Multiple Trauma Respiratory Distress Syndrome, Adult	Drug Inhal [Pul Place
	NCT03808922	STOP PIV - Phase III DAS181 Lower Tract PIV Infection in Immunocompromised Subjects	R	Lower Respiratory Tract Infection Parainfluenza Immunocompromised	Drug Place

	NCT04298060	DAS181 for Patients With Severe Hospitalized Flu and SAD-RVs (COVID-19)	NR	Influenza Infection SAD-RV Infection and COVID-19	Drug Place
	NCT02315898	Inhaled Tissue Plasminogen Activator for Acute Plastic Bronchitis	R	Plastic Bronchitis Protein-Losing Enteropathies Healthy	Drug inhal
Proteins	NCT04204252	Evaluate Efficacy and Safety of "Kamada-AAT for Inhalation" in Patients With AATD	R	Alpha 1-Antitrypsin Deficiency	Drug Antit Place
	NCT02598999	Dose Escalation Study of ALX-009 in Healthy Men and Cystic Fibrosis (CF) and Non-CF Bronchiectasis Patients	R	Cystic Fibrosis Bronchiectasis	Drug OSCN bLF
	NCT04183062	BIO-11006 for Osteosarcoma and Ewing's Sarcoma Lung Metastases	R	Osteosarcoma Metastatic Ewing's Sarcoma Metastatic	Drug (gem doce 1100
	NCT03472053	A Study of BIO-11006 in the Treatment of Advanced Non-Small Cell Lung Cancer	A	Non Small Cell Lung Cancer Stage IIIB	Drug plus care of Ca
	NCT03202394	Evaluation of Safety & Efficacy of BIO-11006 Inhalation Solution in Patients With ARDS	R	Respiratory Distress Syndrome, Adult	Drug 1100 Place
	NCT02337270	Phase 1 Clinical Trial of the Safety and Immunogenicity of an Adenovirus-based TB Vaccine Administered by Aerosol	R	Tuberculosis	Biolo Ad5A
	NCT04121494	ChAdOx1 85A Aerosol Versus Intramuscular Vaccination in Healthy Adults (TB039)	R	Mycobacterium Tuberculosis, Protection Against Tuberculosis	Biolo 85A - aeros Chad
	NCT03912207	Investigating Immune Responses to Aerosol BCG Challenge in Healthy UK Adults	R	Tuberculosis	Biolo Danis place
	NCT04545541	Nebulised Heparin in Patients With Severe COVID-19	NR	Covid19 Respiratory Failure	Drug unfra hepa

Table 2. Overview of biologics in pre-clinical development with information on indication for use (condition), animal species, in which it was tested, and summary of findings.

Obstructive lung diseases					
Disease	Classes	Name of biologic	Species	Summary of effects	Reference
Asthma	Peptide	25-mer peptide targeting MARCKS phosphorylation site	Mouse	Decrease in eosinophils, neutrophils, mucus production, collagen deposition, and airway hyperresponsiveness	[139]
Asthma	Peptide	VIP derivate (IK312532)	Rat	Decrease of EPO, MPO, LDH activities in plasma, inflammatory cells in BALF, airway wall thickness, and epithelial hyperplasia	[140]
COPD	Peptide	VIP derivate (IK312532)	Rat	Significant reduction of recruited inflammatory cells	[141]
Asthma	Protein	Bee Venom phospholipase A2	Mouse	Goblet cell hyperplasia, Th2 cytokines, eosinophils, neutrophils, and macrophages in the airways decreased	[142]
Asthma	Protein	Pegylated IL-2 (combined with budesonide)	Mouse	Reduction of airway hyperresponsiveness, and Th2-associated cytokines	[143]
Asthma	Protein	Low molecular weight heparin	Rat	Reduction in pro-inflammatory cytokines, cell number; increased TGF- β levels in BALF, improved	[144]

				histopathology and airway obstruction	
COPD	Protein	Heat shock protein ab-crystallin	Mouse	Decrease of lung infiltration with lymphocytes and neutrophils	[145]
CFMIPMIPMIP	Protein	Lactoferrin	Rat	Reduction of bacterial load, infiltrated leukocytes, and iron overload in the lungs	[146]
CF (P. aeruginosa)	Protein	Lactoferrin	Mouse	Bacterial load, inflammation markers TNF, IFN- γ , and iNOS reduced	[147]
CF (infection)	Peptide	Esculentin peptides	Mouse	Reduction of bacterial burden	[148]
Acute lung injury/ARDS					
Pneumonia (Methicillin-resistant S. aureus)	Peptide	Cathelicidin, LL-37 and innate defense regulator peptide	Mouse	Anti-inflammatory effects	[149]
Pneumonia (P. aeruginosa)	Peptide	Antimicrobial peptide WLBU2	Mouse	Decrease of bacterial load, leukocyte infiltration, and cytokine expression	[150]
Pneumonia (P. aeruginosa)	Protein	Lactonase	Rat	Reduced mortality, bacterial load, and lung damage	[151]
Pneumonia (P. aeruginosa)	Protein	Lactoferrin	Mouse	Reduction of neutrophil recruitment, and pro-inflammatory cytokines	[152]
ARDS	Peptide	HSP70 + HIV-1 TAT cell	Rat	Reduced MPO and lung inflammation	[153]

		penetrating peptide			
ARDS	Protein	IL-6	Mouse	Reduced cytokine levels in BALF, and reduced lung tissue inflammation	[154]
ARDS	Protein	2-O, 3-O-desulfated heparin	Mouse	Inflammation and chemotactic effects reduced	[155]
Pulmonary hypertension	Peptide	Adenomedullin	Rat	Decrease in right ventricular pressure	[156]
Tuberculosis	Protein	Lipokel complex with cutinase-like proteins 1, 6, and MPT83	Mouse	Protective response against M. tuberculosis obtained with Culp 1-6-Lipokel and MPT83-Lipokel	[157]
Tuberculosis	Protein	Bacille Calmette-Guerin	Guinea pig	Reduced bacterial burden and lung pathology	[158]
Tuberculosis	Protein	rAg85B	Guinea pig	Granuloma formation decreased	[159]
Cancer					
Lung cancer	Peptide	Listeria-derived polypeptide	Mouse	Number of tumor nodules reduced	{[160]}

Abbreviations: ARDS, acute respiratory distress syndrome; BALF, bronchoalveolar lavage fluid; CF, cystic fibrosis; EPO, eosinophil peroxidase; IL, interleukin; Hsp70, Heat shock protein 70; ICAM-1, Intercellular Adhesion Molecule 1; iNOS, inducible nitric oxide synthase; LDH, lactate dehydrogenase; LL-37, human cathelicidin; MARCKS, Myristoylated alanine-rich C-kinase substrate; MIP, macrophage inflammatory protein; MMP-2, myeloperoxidase 2; MPO, myeloperoxidase; MPT83, Mycobacterium tuberculosis protein; ROS, reactive oxygen species; TGF- β , tumor growth factor beta; TNF, tumor

necrosis factor; VCAM-1, vascular cell adhesion molecule 1; VEGF, vascular endothelial growth factor; VIP, vasoactive intestinal peptide.