Problems and Examples of Biomolecule Inhalation for Systemic Treatment

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In recent decades a number of biomolecules, mostly peptides and proteins, have come into the focus of interest for inhalant administration as an alternative of parenteral delivery. Novel inhalation devices and optimization of the breathing technique enabled researchers to develop methods for the non-invasive administration of these compounds, especially for treatment of chronic systemic disorders (e.g. diabetes mellitus, anticoagulation). However, beside aspects of aerosol delivery and inhalation, a number of special biochemical and biological aspects must be considered. These include the physical and biochemical stability of the biomolecules within the nebulization process as well as after pulmonary deposition, and the alveolar absorption of the compound.

**Stability of Biomolecules**

Independent from the type and the MW of the biomolecule, aerosolization may result in denaturation and loss of functionality, for instance by oxidation. For example, aerosolization by means of an air-jet nebulizer may cause an inactivation of recombinant human G-CSF (MW: 18.8 kDa), interferon-\(\alpha\) (MW: 19 kDa) and growth hormone (MW: 22 kDa), whereas \(\alpha_1\)-antitrypsin (MW: 51 kDa), desoxyribonuclease (MW: 30 kDa) and heparin (MW: 3–20 kDa) are more stable [1, 2]. For enhancement of stability within the nebulization process, various additives may be added or a dry powder aerosol may be used instead of a liquid aerosol [1, 2].

**Physiological Inhibitors of Absorption**

The lung has been exposed to microorganisms and foreign substances from the environment for millions of years within the evolution process. In consequence, a complex defense system has been developed protecting the respiratory tract from the nostrils down until the alveoli. In
upper airways and bronchi, defense mechanisms consist of anatomic barriers, cough, mucociliary apparatus, airway epithelium, secretory immunoglobulin A, dendritic cell network and lymphoid structure [3]. About 90% of inhaled particles with diameters >2–3 μm are deposited in the central airways on the mucus overlying the ciliated epithelium from where they are rapidly transported to the trachea by means of the mucociliary escalator and then swallowed into the gastrointestinal tract [3–5]. The absorption of biomolecules deposited there is further reduced by the thickness of mucus layer and respiratory epithelium as well as local peroxidases.

Much better conditions for absorption are found in the lung periphery, i.e. the alveoli [1, 2, 6–8]. However, even there a number of defense mechanisms exist (fig. 1). The first barriers after contact are the mucus layer (a complex mixture of lipids and glycoproteins, but also surfactant from the lower respiratory tract) and the alveolar lining fluid (includes a large amount of surfactant with phospholipids and surfactant apolipoproteins acting as a surface-active substance). Amount, composition and thickness of the mucus layer depend on its localization in the respiratory tract, inflammatory and neuronal factors whereas synthesis and release of surfactant from type II pneumocytes are modulated by hyperventilation, endogenous and exogenous factors (pharmaceuticals) [1, 2]. Cells located in the respiratory tract (mostly macrophages representing about 85% of cells retrieved by BAL in healthy individuals) also counteract the absorption of deposited substances. They serve as an unspecific defense mechanism (e.g. against bacteria and inhaled particles) and act via phagocytosis, secretion of reactive oxygen species by means of respiratory burst and release of mediators of inflammation. However, even granulocytes (about 1–2% in normal BAL) may invade rapidly and serve as potent inhibitors of absorption, e.g. by phagocytosis, respiratory burst and secretion of proteases. Last but not least, lymphocytes (10–20% in normal BAL, mostly CD4+ lymphocytes) play a crucial role in the immunological response after antigen presentation by macrophages and dendritic cells. However, lymphocytes can also phagocytose and include granules containing proteases and proteolytic enzymes [1–3]. Type I pneumocytes which cover about 97% of the alveolar surface (the remaining area consists of type II pneumocytes) express carboxypeptidase which degrades a number of peptides and proteins. However, the total distance between the respiratory tract and circulation is only 0.5 μm facilitating the diffusion of gases as well as penetration and transport of fluids and (inhaled) macromolecules. The latter can pass alveolar epithelium via different transport mechanisms, which are intracellular tight junctions, membrane pores and vesicular transport by type I and type II pneumocytes (fig. 1) [1, 2].

Another transport mechanism serving for the exchange of fluids and macromolecules are membrane pores. It is assumed that pores of different sizes exist, which can increase their diameter in case of an existing hydrostatic pressure gradient [1, 2].

In pneumocytes types I and II another mechanism of vesicular transport has been described, which is comparable to that in epithelial and endothelial cells. In detail, the vesicular transport mechanism of type I pneumocytes is pressure-independent and allows the transcellular transport of fluids and macromolecules. However, an estimation of the functional capacity of this transport mechanism is difficult, because (1) the number of vesicles increases in liquid-filled lung indicating their role in the transport of fluids, (2) the glyocalix affects the uptake of proteins via specific or unspecific binding mechanisms and a number of receptors and binding proteins were identified on capillary endothelia, (3) the definite processing of the vesicles inside the cells and the mechanisms for their movement (e.g. Brownian movement) are not conclusively identified, (4) the energetic mechanisms of membrane displacement and fusion of the vesicles are not yet conclusively
Fig. 1. Barriers for absorption of peptides and proteins after peripheral/alveolar deposition [1, 2].
elucidated, and (5) different types of vesicles (e.g. clathrin-coated and clathrin-uncoated) exist, which both play a role in transcytosis, but differ in respect to their characteristics of protein uptake [1, 2].

Surfactant produced from pneumocytes type II together with proteins plays an important role for the clearance of macromolecules by means of the alveolar lining fluid. Further cellular processing can take place with or without binding of the macromolecules on the cellular surface and depends strongly on the charge of the molecules. A large proportion of the material absorbed by endocytosis from type II pneumocytes is deposited in lamellar bodies. In addition, transcellular transport represents another mechanism for absorption of macromolecules [1, 2].

The basal lamina (thickness of about 20–25 nm) placed below the epithelium predominantly consists of glycoproteins (e.g. laminin and fibronectin) and has an anionic charge on its outer surface. Presumably, the latter regulates the permeation dependent on size and charge of the molecules. After their passage through the alveolar wall and alveolar basal lamina, inhaled substances reach the interstitium, where proteins can be bound by macromolecules or inactivated or phagocytosed by macrophages or transported to the lymphatic system. In the latter case, proteins can be detected after some hours in the circulation. Endothelial basal lamina and endothelium are also barriers for the absorption of macromolecules. However, compared to the other barriers described before they are less effective in inhibiting the absorption of biomolecules (fig. 1) [1, 2].

Factors Affecting the Absorption of Macromolecules

After alveolar deposition, proteins with a low MW are absorbed more rapidly than those with a high MW. The bioavailability of proteins with a MW up to 30 kDa (which includes the vast majority of proteins used in clinical therapy) is between 20 and 50%. However, because of proteolytic degradation the bioavailability of some proteins is much smaller [1, 2, 7–10]. Other variables affecting the absorption are pH value, electrical charge, surface activity, solubility and stability in the alveolar environment [1, 2, 7, 9]. In hydrophilic compounds (e.g. carbohydrates, peptides and proteins) the half-life time of alveolar absorption (t0.5) as well as the time to reach the maximum serum concentration (tmax) increase as a function of their MW [2, 9, 10].

Improvement of Macromolecule Absorption

Biomolecules deposited in the alveoli can be absorbed by four distinct mechanisms: phagocytosis by alveolar macrophages, paracellular diffusion via tight junctions, vesicular endocytosis or pinocytosis, and receptor-dependent transcytosis [1, 2, 8]. Accordingly, the functional role of barriers and transport mechanisms and their control by physiological and pharmacological factors is very different and in consequence a large number of very different compounds and techniques for absorption enhancement were investigated [1, 2, 8, 11], some of which are described below in more detail.

Even though the activity of proteases and peptidases in the alveolar region is much lower than in the gastrointestinal tract, proteolytic degradation of susceptible proteins can cause a relevant reduction of the bioavailability [9]. Therefore, bioavailability can be increased by addition of protease inhibitors (e.g. nafamostat mesilate, aprotinin and p-amidinophenylmethanesulfonyl fluoride•HCl). However, the effects of the various protease inhibitors are very specific to the type of the protease or peptidase [2, 6, 10, 11].

The heterogenous group of surface-active compounds (e.g. bile acids, fatty acids, non-ionic detergents) is assumed to increase the alveolo-capillary transport by an interaction with the cell
membrane resulting in a liquefaction followed by an increased permeability and/or a modulation of cellular tight junctions followed by an increased paracellular permeability. Presumably, bile acids increase the absorption by alteration of the mucus layer, protection of proteins against enzymatic degradation, disaggregation of protein multimers, opening of epithelial tight junctions as well as solubilization of phospholipids and proteins out of the cell membrane followed by formation of micelles. However, a strong absorption-enhancing effect can result in damage to the epithelial surfaces, especially after treatment with higher doses and longer treatment times [2, 10, 11].

Cyclodextrins are cyclic polymers of glucose, which can form complexes with molecules fitting into their lipophilic inner structure. The underlying modes for absorption enhancement are solubilization and complexation of membrane lipids and proteins of epithelial cells, inhibition of proteolytic enzymes and modification of the physicochemical properties (e.g. solubility and partition coefficient) of the administered substances. However, the toxicity increases with the intensity of absorption enhancement, both depending on the structure of the compound [2, 6, 11].

Based on the observation that smaller particles are more rapidly phagocytosed than larger ones, methods were developed to bind macromolecules to microparticles [6, 9]. For this purpose, proteins are packed into the inner of biologically degradable polymers or lipids. This results in a reduction of physiological clearance in the alveolar region and proteolytic degradation of proteins after phagocytosis by alveolar macrophages. In addition, a sustained release of the compounds from the microparticles is achieved [9, 10]. Microparticles for drug administration can be classified into porous particles and liposomes [2, 6, 9–11]. Pharmacological properties of porous particles depend on the used material, particle size, porosity and surface structure, whereas those of liposomes depend on particle size and chemical properties (charge, MW) of the consisting phospholipids [2, 6, 11].

Liposomes are particles (size range from some nanometers up to a few micrometers) consisting of hydrophobic lipids and phospholipids forming a closed, concentric, bilayer-membrane vesicle with a hydrophilic aqueous center [2, 11, 12]. According to this structure, both hydrophobic and hydrophilic compounds can be packed into liposomes prior to transportation into the lung. Hydrophilic compounds (e.g. pharmaceuticals and larger biomolecules) are entrapped into the vesicle in the inner of the liposome, whereas lipophilic (hydrophobic) compounds are encapsulated into the membrane bilayer. Because of their strong chemical and structural similarity, liposomes merge with cell membranes and facilitate drug delivery into the interior of the cell (fig. 2). However, especially small liposomes are also absorbed via cellular phagocytosis [12]. Depending on their structure, liposomes have a high transport capacity and allow the transport of a large number of very different compounds. One more characteristic is the sustained release of the compounds transported by liposomes [2, 10–12]. Even though the majority of studies revealed no toxic effect of liposomes, it must be considered that both effects, absorption enhancement and lung toxicity, depend on their physicochemical properties (concentration, charge, chain length and MW of phospholipids) [2, 6, 11, 12].

Another approach is the modification of proteins by fusion to the Fc domain of an IgG1 (IgG subtype 1) [2, 13, 14]. In contrast to rodents where the expression of the Fc receptor in the gut rapidly decreases after weaning and remains low in tissues of adult animals, the Fc receptor in humans keeps expressed in several absorptive tissues (e.g. lung, kidney, intestine) even in adulthood. IgG Fc fusion proteins are taken up into epithelial cells by pinocytosis. In detail, a coated vesicle is formed by invagination of the plasma membrane entrapping IgG and other solutes in its lumen. Obviously, only a small proportion of IgG binds
to FcRn at the plasma membrane, whereas most of the binding takes place intracellularly, because the majority of FcRn is localized in acidic endosomal vesicles inside the cell. The transport vesicles containing IgG bound to FcRn do not fuse with degradative lysosomes but rather pass unidirectionally through the epithelial cell, driven by the pH gradient between luminal and serosal exposures of the epithelial cells. As the binding of IgG to FcRn is pH-dependent (tight binding at slightly acidic pH), there is a release of IgG from FcRn after fusion of the transport vesicles with the plasma membrane at the basolateral site of the epithelial cells because of the neutral to slightly alkaline pH value of the interstitial space. Passage of IgG into the circulation is most likely primarily paracellular because of the absence of tight junctions between endothelial cells. The FcRn receptor is also responsible for the long half-life time of IgG in the bloodstream, because it protects IgG from degradation. As in epithelial cells, IgG is taken up from vascular endothelial cells by pinocytosis. However, in contrast to epithelial cells, IgG there is not subject of transcytosis, because the endocytic vesicles containing IgG bound to FcRn return to the plasma membrane of the endothelial cells, so that IgG is released back into the bloodstream resulting in a recycling process.

**Fig. 2.** Acceptance of a liposome into a cell. Liposomes consist of lipids and phospholipids [2, 12]. Each phospholipid has a polar hydrophilic ‘head group’ and two hydrophilic ‘tails.’ When phospholipid molecules are hydrated under low-shear conditions they spontaneously arrange themselves in sheets with their heads up and tails down. These sheets then join tails-to-tails and form a bilayer membrane that encloses water and – if added – water-soluble compounds (e.g. pharmaceuticals and larger biomolecules) in the center of the sphere. If liposomes come into contact with cell membranes consisting of phospholipids, lipids and proteins, the liposome membrane fuses with the cell membrane facilitating the entry of the encapsulated drug into the interior of the cell.
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for IgG protecting IgG from lysosomal degradation. Fc fusion proteins can be efficiently administered as liquid aerosols and several studies have demonstrated a good tolerability, a high bioavailability even of larger proteins (e.g. erythropoietin) and an increased half-life time in the circulation in animals or humans [2, 13, 14].

**Systemic Treatment with Inhaled Macromolecules**

A number of studies investigated the feasibility of macromolecule inhalation for systemic treatment. A focus was set on hormones (insulin, calcitonin, growth hormones, somatostatin, thyroid-stimulating hormone, and follicle-stimulating hormone), growth factors (G-CSF and GM-CSF), different interleukins (e.g. IL-2) and heparin (unfractionated and LMWH) [6, 15–18]. However, in humans, most data are available for insulin, heparin and IL-2. In the following, some examples, except insulin which is described elsewhere in this book, are compiled.

Heparin, an acidic sulfated mucopolysaccharide, is characterized by a MW of unfractionated heparin between 2.8 and 58 kDa (mean value: 15 kDa) and between 2 and 6 kDa in case of fractionated LMWH. Both require parenteral administration and serve as an anticoagulant due to their binding to antithrombin III resulting in a conformation change of this protein. Beside this, a lot of other properties of heparin (e.g. interaction with growth factors, regulation of cell proliferation and angiogenesis, modulation of proteases and antiproteases) are of interest in medical research.

Since 1965, a number of studies had investigated safety and feasibility of the inhalation of heparin and LMWH for anticoagulation. It was found that inhalant administration was effective, well tolerated and not followed by relevant pulmonary or systemic side effects [16, 19]. However, some studies showed a strong variability of the anticoagulative effect, e.g. due to an inadequate diameter of the produced aerosol particles in older nebulizers and a not sufficiently standardized inhalation maneuver. A more recent study investigated the anticoagulative effect (determined by measurement of the anti-FXa activity) of different doses of inhaled certoparin in healthy humans in comparison to subcutaneous administration. The study revealed a rapid onset of the anticoagulative effect after certoparin inhalation, a decrease of the interindividual variabilities after administration of higher doses (up to 9,000 IU) when compared to lower doses, a satisfactory anticoagulative effect after inhalation of 9,000 IU and a longer duration after inhalation of 9,000 IU when compared to subcutaneous administration of 3,000 IU which was achieved without side effects [16].

Erythropoietin, an erythrogenic growth factor (epoetin α: MW: 14.7 kDa; epoetin β, γ, δ, ε, ω: MW: 18.2 kDa) is used for stimulation of erythropoiesis in the bone marrow, e.g. in patients with end-stage renal failure and cancer. Due to its large MW the pulmonary absorption without methods for absorption enhancement is low [2, 14]. An interesting method based on Fc fusion proteins has been developed to improve pulmonary uptake and pharmacokinetics of erythropoietin (and also other proteins) [2, 13, 14]. In brief, in cynomolgus monkeys a better absorption of Epo-Fc dimers was observed after a shallow breathing pattern than after a deep inhalation due to the higher expression of Fc receptors in the central airways. After inhalation of the Epo-Fc dimer, the Epo-Fc monomer and unconjugated erythropoietin bioavailabilities of 5%, 35% (which is similar to that after subcutaneous administration) and 15% were observed, respectively. The low bioavailability of the Epo-Fc dimer was assumed to be caused by its higher MW or steric hindrance of IgG and erythropoietin. Independent from the mode of administration (inhalation, intravenous administration) the observed plasma half-life times (t0.5) were higher for the Epo-Fc dimer and the Epo-Fc monomer than for unconjugated erythropoietin, demonstrating an increase of t0.5 due to the
fusion to the Fc residual of the immunoglobulin. Functional analysis by measurement of reticulocytes in blood revealed that all types of inhaled erythropoietin and both fusion proteins were biologically active, although there were differences with respect to the strength of the reticulocyte-increasing effect [13, 18]. Some further experiments including measurement of pharmacokinetics after administration of different doses were also performed in healthy human volunteers and confirmed the absorption and a dose-dependent biological effect after inhalation of the Epo-Fc fusion protein [14, 18].

IL-2 is produced from T lymphocytes after antigen stimulation and serves as an immune modulator, e.g. activating cytotoxic T cells and natural killer cells making it an interesting target in tumor research [20]. It was observed that IL-2 caused a suppression of metastases of malignant tumors, especially metastases from melanoma and kidney cancer. In the latter, persisting remissions were achieved in a number of patients. In consequence, IL-2 treatment received approval from the Food and Drug Administration (FDA) for treatment of metastasizing kidney cancer and melanoma in 1992 and 1998, respectively [20]. Most frequently, IL-2 was subject of systemic administration, however in a number of studies there was also an inhalant administration of IL-2 liposomes alone or in combination with other cytokines (e.g. interferon-α). Most data were published for patients with advanced kidney cancer, especially those with pulmonary or mediastinal metastases [20–23]. The inhalation (first-line and second-line therapy) was well tolerated showing fewer side effects than systemic treatment with cytokines and was followed by a relevant increase of survival time. Inhalation of high IL-2 doses (second-line therapy) was also followed by temporary regression of pulmonary metastases but not extrapulmonary metastases in patients with melanoma [21, 22].

Treatment with recombinant GM-CSF (MW: 14.6 kDa) has been approved for therapy of patients to recover neutrophils (e.g. after induction chemotherapy for treatment of acute myelogenous leukemia, mobilization of hematopoietic progenitor cells prior to cytopheresis and following transplantation of hematopoietic progenitor cells) [23]. However, several other potential clinical indications, e.g. use in antitumor therapy and treatment of alveolar proteinosis, have been investigated [23]. The glycoprotein is usually administered parenterally but in studies for treatment of cancer or alveolar proteinosis it has been administered by means of inhalation [18, 23]. The feasibility of aerosol administration of GM-CSF was proven in cynomolgus monkeys more than 15 years ago [18]. A number of studies investigated the effect of GM-CSF on pulmonary alveolar proteinosis which is an orphan disease (less than 500 reported cases until 2006 and first described in 1958). Since a first case report from 1996, several investigators demonstrated that inhalant administration of GM-CSF aerosol alone or if necessary in combination with whole lung lavage is a safe and efficient therapy for treatment of pulmonary alveolar proteinosis even for a longer treatment period resulting in a therapy-dependent improvement of clinical behavior and lung function parameters [18, 24, 25]. Other studies investigated the effect of various study regimens and doses of inhaled recombinant GM-CSF on different types of cancer, e.g. metastases of kidney carcinoma, osteosarcoma and melanoma [18]. Usually, inhalation of lower doses was not followed by relevant changes of lung function and side effects. In addition, there were also only minor increases of white blood cells and neutrophils under therapy. However, treatment with aerosolized GM-CSF resulted in a relevant disease regression or reduction of disease progression in some patients [18]. In a more recent study, escalating doses of up to 2,000 μg b.i.d. were administered in patients with lung metastases of melanoma. The investigators observed an acceptable toxicity of inhaled GM-CSF, the greatest changes of antitumor immunity in patients receiving the highest drug doses and
longer times of progression-free survival in patients developing an immune response [26].

Cyclosporin A, a cyclic peptide (MW: 1,200 Da) serves as an immunosuppressant for prevention of graft rejection in patients after organ transplantation as well as in patients with autoimmune diseases [27]. In a number of studies predominantly performed in lung transplant recipients, the effect of cyclosporin liposome inhalation was investigated [27]. After inhalation the lipophilic compound is rapidly absorbed. However, the pharmacokinetics indicate a temporary uptake of the compound by alveolar macrophages as well as its interaction with pulmonary surfactant or membranes of alveolar epithelium [28]. Depending on the deposited lung dose of inhaled cyclosporin A, an improved transplant function (determined by measurement of the forced expiratory volume in 1 s) was observed in lung transplanted patients with graft rejection. At the same time, patients treated with aerosolized cyclosporin A required lower doses of other immunosuppressants when compared to the control group. However, inhalant immunosuppressive therapy was well tolerated and there were no higher rates of pulmonary infections as well as no hepatotoxic or nephrotoxic effects [29]. More recent data of the same study group demonstrated that deposition of sufficient pulmonary doses of cyclosporin A can prevent the decrease of graft function and the occurrence of bronchiolitis obliterans (which is largely affecting the long-time prognosis after lung transplantation) and in consequence improve the long-time outcome in lung transplanted patients [30].

Safety of Macromolecule Inhalation

Safety and tolerability of pulmonary administered compounds can be largely different from those after subcutaneous administration. Inhaled pharmaceuticals as well as additives for absorption enhancement may induce an incompatibility (e.g. immunization in case of proteins and induction of specific effects on the target organ lung in case of hormones) as well as damage of lung epithelium directly (e.g. bile acids, cyclodextrins and other absorption enhancers) or via production of reactive oxygen species (e.g. in case of cationic liposomes). Last but not least, pulmonary diseases may complicate or prevent inhalant drug therapy under some circumstances [2, 6, 11, 17].

Conclusions

In recent decades, inhalation of biomolecules has come into the focus of interest. However, prior to studies investigating the inhalation of these compounds, the physical and physiological background for reproducible administration of sufficient drug doses into the lung had to be elucidated. After solving these basic questions, the feasibility of inhalative administration was investigated for a large number of biomolecules (mainly peptides and proteins). However, up to now, data regarding the long-time effects of inhaled macromolecules except insulin and heparin are sparse. In addition, there are also few data regarding the feasibility and safety of carriers (e.g. microparticles and liposomes) as well as stabilizers and absorption enhancers for pulmonary drug administration. Therefore, future studies are required for further investigation of long-time effects and optimization of inhalative drug administration. Then it is likely that inhalation-based methods for drug administration will serve as a safe and convenient alternative of subcutaneous injection in patients with systemic diseases.
References