INTRODUCTION

Since about 25 years, a number of methods for recombinant synthesis of peptides and proteins were developed allowing the production of large amounts of these compounds (e.g., growth factors, hormones, monoclonal antibodies, and cytokines) which play an important role in clinical treatment (1). A strong disadvantage lies in their biochemical properties (high molecular weight, hydrophilicity, sensitivity against chemicals, and proteolytic enzymes). Therefore, these compounds cannot be administered orally, but require parenteral administration. As some of these substances (e.g., insulin) are required for the treatment of chronic diseases, this type of drug administration has negative effects on convenience and compliance of the patients. Several alternative methods for drug application have been developed in order to solve this problem (1). Inhaled application (via nose or mouth) even of high molecular weight compounds seems to be a method of choice. However, several preconditions must be fulfilled to allow administration of adequate and reproducible drug doses. These are biophysical and physiological factors (e.g., aerosol particle size, and breathing maneuver (inspired volume, inspiratory flow, and end-inspiratory breath holding time), which are described in more detail in other reviews (2, 3) as well as the physical and biochemical stability of the pharmaceutical compounds designed for aerosolization (aqueous solution, dry powder, and suspension or solution in propellants (4, 5).

Within the evolution process millions of years ago, a complex defense system has been developed for protecting the respiratory tract against microorganisms and environmental noxes, from the nostrils down until the alveoli. Defense mechanisms of upper airways and bronchi are anatomic barriers, cough, mucociliary apparatus, airway epithelium, secretory immunoglobulin A (IgA), dendritic cell network, and lymphoid structure (6). It is very effective, as about 90 % of inhaled particles with diameters larger than 2-3 µm are deposited in the central airways on the mucus overlying the trachea by means of the mucociliary escalator and swallowed into the gastrointestinal tract (see Fig. 1). Furthermore, the thickness of mucus layer and respiratory epithelium and also the peroxidases reduce the absorption of biomolecules deposited in the central airways. Much better conditions for absorption of inhaled macromolecules are found in the lung periphery. Firstly, the size of the alveolar surface depends on the distension of the lung and varies between 80-140 m², which is about half of a tennis court (132 m²) and much larger than that of the nose (about 180 cm²) (4, 7, 8). Secondly, the thickness of the lung epithelium in most regions is between 0.1-0.2 µm (9, 10) resulting in a total distance between epithelial surface and blood between 0.5-1.0 µm (8), which is much less than in the bronchial tract, where the deposited substances have to pass a distance of 30-40 µm and more between mucus surface and blood (see Fig. 2) (8, 9). Furthermore, the lung is perfused with a blood volume of about 5 l/min at rest (11) without a first-pass effect, which plays a large role for orally administered drugs even though some metabolism takes also place in the lung (1, 4, 8, 10, 12, 13). However, even in the lung periphery a number of defense mechanisms exist, which inhibit the absorption of biomolecules, e.g., microphage uptake.

Clinical experience since many years has shown that aerosol inhalation is an established route for the treatment of pulmonary diseases. In contrast, treatment of systemic diseases by means of aerosol inhalation is a novel therapeutic approach. This was caused for a long time by a lack of accuracy, efficiency, and reproducibility of the administered drug doses due to a poor knowledge of the physiological background of aerosol inhalation, an insufficient inhaler technology as well as a suboptimal breathing procedure. However, these problems have been solved in the last years and nowadays modern aerosol delivery systems allow the production of an aerosol with a defined and optimised particle size combined with an optimized breathing maneuver and optimization of the efficacy of the technology. Clinical studies demonstrated that only a small number of morphological factors (e.g., exogen allergic alveolitis, active sarcoidosis, active smoking) influence alveolar drug deposition and the inhaled systematically active compounds caused no relevant allergic reactions even after inhalation for longer time periods. Up to now, most data are available for the inhalation of insulin which has been introduced in clinical treatment for a short time. However, a lot of other molecules have been tested in aerosol inhalation studies. This review describes some examples other than insulin in the field of inhalant treatment of systemic diseases.

Key words: aerosol therapy, inhalation, systemic treatment, peptides, proteins
METHODS FOR ABSORPTION IMPROVEMENT

Various enzymes, especially peptidases and proteases, degrade macromolecules, especially peptides and proteins, by proteolysis. Addition of absorption enhancers to these pharmacological compounds considerably increases the absorption after their deposition in the respiratory tract. Prevention of proteolysis by addition of protease inhibitors or packing of the macromolecules into particles can further increase the bioavailability (5, 14-18). Packing into microparticles can also be used for the development of ‘sustained release’ pharmaceuticals. However, all these substances for absorption enhancement which are described in more detail in other reviews do not only affect the pharmacological properties of the administered macromolecules (e.g., bioavailability, time to reach the maximum plasma concentration (tmax) and maximum plasma concentration (Cmax)), but also have an own active profile and toxicity (1, 15, 16, 19).

EXAMPLES FOR THE INHALATION OF BIOMOLECULES

Feasibility and safety of pulmonary administration of drugs and biomolecules for systemic treatment has been demonstrated in a large number of studies. However, there are only little data regarding the long time effects of inhaled macromolecules except insulin and heparin (1, 8, 11, 12, 17, 20-26). In the following, a number of examples, except insulin and heparin, which were or will be subject of other reviews (17, 27) is given.

Growth hormone

Since about 50 years replacement of human growth hormone (hGH; MW: 22100 Da) is used to promote growth in children with pituitary dwarfism due to GH deficiency (28-30). However, even GH-deficient adults are treated in order to restore/ameliorate abnormalities of energy metabolism (carbohydrates and lipids), body composition, muscle mass and strength, cardiovascular structure and function as well as bone metabolism and density (28, 31-33). At the beginning, treatment was performed by means of injection with extracted hGH, but since about 1981 recombinant human growth hormone (rGH) is used in clinical practice (28). As the treatment is performed for a longer time period (up to years), a non-invasive administration (e.g., intranasal, inhalant) would largely improve the compliance of the patients. However, the alternative routes of drug administration were up to now experimental and suffer from specific problems. For instance, intranasally administered rGH requires absorption enhancers to achieve sufficient plasma concentrations and may cause nasal sensations (34). Furthermore, in case of inhalant therapy hGH tends to aggregate during nebulization and therefore requires stabilization, e.g., by addition of detergents or administration of a powder aerosol (4, 35).

The bioavailability of growth hormone after pulmonary administration in different studies is between 5% and 45% (36-39). For instance, Folkesson et al. (38) observed plasma levels of 6.0±1.7% and 3.3±1.2% (male and female rats, respectively) of the dose administered by intratracheal instillation. The plasma level did not increase linearly as a function of the administered dose, reached the maximum within 1 h after administration and almost disappeared after 24 h. The clinical effect of the administered GH was demonstrated by the increase of the body weight of the rats under therapy. In contrast, Patton et al. (39) observed a bioavailability of 36% after intratracheal instillation of recombinant methionyl human growth hormone in rats. The maximum serum concentration was observed 6 h after administration and after 24 h about 70% were no more detectable in the lungs. The investigators also performed histological investigations and found that pulmonary macrophages were stained for growth hormone demonstrating that these cells play a role in the degradation of the hormone after pulmonary administration. In another study Colthorpe et al. (37) investigated the deposition and pharmacokinetics of hGH administered by aerosol and instillate. The bioavailability was higher after aerosol administration than that after instillation (45% vs. 16%), which was explained by a more peripheral deposition of the aerosol (enhancing the absorption) and a differential effect of mucociliary clearance (rapid clearance in the larger airways). The observed post-peak half-life times were longer after pulmonary than after intravenous administration (347, 578, and 40 min for
Aerosol, intratracheal and intravenous administration, respectively). Furthermore, the apparent absorption rate constants resulting from instillation and aerosolization were equivalent (0.0012 and 0.0020 min⁻¹, respectively); however lung-to-blood transfer rate constants for aerosol delivery were greater than for instillation (0.00071 vs. 0.00018 min⁻¹) (37). In a more recent study Bosquillon et al. (36) investigated absorption and pharmacokinetics of human growth hormone administered by insufflation of a spray-dried powder aerosol (formed with lactose and dipalmitoylphosphatidylcholine) and an intratracheal spray-insufflation of a solution of the hormone in comparison with subcutaneous and intravenous administration. A more rapid absorption, a higher bioavailability and a larger area under the plasma concentration vs. time curve (AUC) were found after insufflation compared with spray-instillation (tₚ₅₀: 23 vs. 51 min, 23±2 vs. 8±1% and 19520±1436 vs. 6320±1026 ng min/ml, respectively). The authors further performed immunological investigations and observed an intense uptake of fluorescein isothiocyanate labelled hGH (FITC-hGH) by alveolar macrophages within 1 hour after delivery.

**Calcitonin**

Treatment with human or salmon calcitonin (MW: 4500 Da) can be performed for clinical therapy of very different diseases, e.g., osteoporosis, Paget’s disease of the bone, and central giant cell granuloma. However, modern treatment of osteoporosis is predominantly based on the administration of bisphosphonates, vitamin D, and calcium, all of which can be easily given orally. The clinical feasibility of intranasal delivery in part with absorption enhancers and stabilizers (e.g., gelatin, N-acetyl-L-cysteine, and sodium tauro-24,25-dihydrofusidate), from which some may cause mucosal irritation, have been demonstrated in several studies performed in animals and patients (34, 40-42). Further studies investigated the pulmonary administration of calcitonin. However, the pulmonary administration of the peptide requires measures for improving its stability prior to nebulization and its pulmonary absorption by addition of absorption enhancers and protease inhibitors. For instance, an improved stability of the powder aerosol can be achieved e.g., by addition of mannitol alone or in combination with albumin and citric acid/sodium citrate prior to spray-drying (43, 44). In an early study, Kobayashi et al. (45) investigated the effect of absorption enhancers and protease inhibitors on the absorption of calcitonin solution after intratracheal administration in rats. It was observed that the biological effect of calcitonin has a rapid onset and increased as a function of the administered dose. As expected, the effect was smaller after intratracheal administration than after intramuscular injection (bioavailability about 30%). Some absorption enhancers (sodium oleate, oleic
acid, sorbitan trioleate, polyoxyethylene (POE) sorbitan monooleate, POE sorbitan trioleate, POE oleyl ether, palmitoleic acid, and linoleic acid) significantly increased the pulmonary absorption of biologically effective calcitonin whereas others (oleyl alcohol, glycerol trioleate, ethyl oleate, palmitic acid, and stearic acid) failed to have a significant effect. Strong differences were also found for absorption enhancers, from which some (bacitracin, chymostatin, potato carboxy peptidase inhibitor (pCPI), phosphoramidon, diisopropyl fluorophosphates (DFP), analogic, Tos-Lys-chloromethylketone (TLCK), and leupeptin) significantly improved the biological effect of the administered calcitonin, whereas others (aprotinin, benzamidine, p-amidinophenyl methane sulfon fluoride HCl (p-APMSF), bestatin, foroxymithin, phenanthroline, amastatin, pepstatin, and elastinal) showed no significant effect. The known specific effect of protease inhibitors on proteases and peptidases (e.g., serine proteases and metalloenzymes) may serve as a possible explanation of the observed differences in their efficacy (45).

In another study, the same investigators studied the effect of different doses of absorption enhancers (oleic acid, lecitin, citric acid, taurocholic acid, dimethyl-β-cyclodextrin, and octyl-β-D-glucose) on the absorption of intratracheally administered calcitonin in rats. The biological effect of the administered calcitonin was higher for powder aerosol than for liquid aerosol and higher after administration of higher doses of the absorption enhancer (46). The effect of different protease inhibitors on the absorption of calcitonin in rats was also investigated by Morita et al. (47). The investigators observed a bioavailability of only 2.7% after administration of calcitonin alone. However, co-administration of sodium glycocholate, bacitracin, and nafamostat mesilate, but not soybean trypsin inhibitor (STI) and bestatin, improved the bioavailability of calcitonin solution administered intratracheally, as determined by the calcium lowering effect. A maximum effect was observed in presence of 20 mM bacitracin, which resulted in an effect similar to that after intravenous administration (47). However, Patton et al. (48), while investigating different biomolecules observed an absolute bioavailability of 17% (determined by radioimmunnoassays) and a peak serum concentration about 15 min after intratracheal administration of both human and salmon calcitonin solution in rats. Komada et al. (49) also investigated the absorption of calcitonin powder aerosol in rats following intratracheal administration and observed a rapid increase of the calcitonin serum concentration reaching Cmax few minutes after administration. However, the plasma concentration also rapidly decreased resulting in a bioavailability of the peptide of 11.5% only. In a later study, Morimoto et al. (50) investigated the absorption of salmon calcitonin bound to gelatine microspheres after intratracheal administration. The authors observed a rapid release of calcitonin from the microspheres and a rapid onset of the hypocalcemic effect within 2 h after administration. The bioavailability of calcitonin depended on size and electrical charge of the microparticles and was highest in positively charged smaller particles (size and charge; 10.9 μm, neg.: 35.4%; 3.4 μm, pos: 51.3%; 11.2 μm, pos: 49.2%; 22.5 μm, pos: 38.2%). More recently, Yamamoto et al. (51) investigated the biological effects of elcalcitolin with and without absorption enhancers in guinea pigs. The experiments were performed with aerosolized calcitonin solution and with DL-lactide glycolide copolymer (PLGA) nanospheres modified with chitosan or not. It was observed that the calcium lowering effect of the chitosan-modified nanospheres was more pronounced and had a significant longer duration (up to 48 h after inhalation) when compared with the effect after administration of the solution or the nanospheres without the modification. Most likely, the difference was caused by an improved mucoadhesion and opening of intercellular tight junctions due to the addition of chitosan. The effect of pegylation by means of different molecular weight polyethylene glycol (PEG; 1, 2, 5 kDa) was recently investigated by Youn et al. (52) in rats treated with an intratracheal instillation of salmon calcitonin. PEGylated and non-PEGylated calcitonin showed a rapid increase of the calcitonin plasma concentration and the calcium lowering activity of the hormone. Dependent on the molecular weight of PEG (unmodified, 1, 2, 5 kDa) the pharmacological parameters plasma half-life time (t 1/2: 34.6, 53.9, 100.7, and 119.4 min, respectively), maximum plasma concentration (Cmax: 54.8, 78.1, 102.9, and 115.2 ng/ml, respectively) and area under the concentration vs. time curve (AUC, 3425.1, 8309.8, 15342.1, and 24873.5 ng min/ml, respectively) increased, demonstrating improved pharmacological properties. The largely increased stability of PEGylated calcitonin in lung homogenates depended also on the molecular weight of PEG. However, a reduced intrinsic bioactivity was found for calcitonin PEGylated with 5 kDa PEG (~30% vs. ~80%) compared with the other types of calcitonin tested, which should be considered while optimising the therapy with peptides modified by means of PEGylation (52).

Although a number of animal studies were performed in the past, studies in which calcitonin was administered in humans are up to now sparse. Deftos et al. (53) investigated the effect of two different powder aerosols of different doses (1 puff, 160U and 2 puffs, 320U) after pulmonary administration in comparison with intramuscular injection of 100U in ten normal male volunteers aged 25 to 90 years. The investigators observed a rapid onset of action resulting in an increase of the serum calcitonin and a decrease of ionised calcium in serum after inhalation and intramuscular injection, respectively. Compared by dose, intrapulmonary administered salmon calcitonin had 66% of the bioactivity and 28% of the bioavailability of the intramuscularly administered compound.

Parathyroid hormone

Parathyroid hormone is a polypeptide consisting of 84 amino acids (MW: 9400 Da; PTH1-84) and is synthesised in the chief cells of the parathyroid gland. Its physiological role is the regulation of calcium homeostasis and bone turnover. However, even the 1 to 34 N-terminal fragment (MW: 4300 Da; PTH1-34) of the hormone exhibits full biological activities. It was demonstrated in clinical studies that administered parathyroid hormone stimulates formation of new cortical and trabecular bone and improves bone growth and density, e.g., in females suffering from postmenopausal osteoporosis (54). In an early study Patton et al. (48) investigated the bioavailability of PTH1-84 and PTH1-34 after intratracheal instillation in rats in comparison with intravenous administration of the hormone. The serum concentration of PTH1-84 showed a peak after 15 min, whereas no clear serum peak was observed for PTH1-34. Accordingly, the absolute bioavailability was higher for PTH1-34 (40%) than for PTH1-34. The effect of PTH powder aerosols of different composition (PTH, albumin, lactose, trehalose, and dipalmitoylphosphatidylcholine (DPPC)) after intratracheal administration in rats was investigated by Codrons et al. (54). The investigators observed no acute inflammation in the lung up to 48 h after inhalation. After inhalation there was a rapid increase and decrease of PTH plasma concentration which is essential because a pulsatile profile results in a net gain of bone mass, whereas a continuous infusion results in a net loss (55, 56). However, there were differences of the pharmacokinetic pattern depending on the particle composition. In detail, the absolute bioavailability of the albumin containing powder was 21% and was increased to 34% after withdrawal of albumin demonstrating that this excipient markedly decreases systemic absorption through binding of the hormone (54). In another study Codrons et
al. (55) investigated the impact of aerosol formulation and pulmonary delivery on the absorption of PTH$_{1-34}$ also in rats. It was observed that addition of DPPC to the particle, even though this compound is abundantly present in pulmonary surfactant, results in an increased absorption and that PTH$_{1-34}$ is better absorbed after deep pulmonary delivery.

**Thyroid-stimulating hormone**

Few studies on the inhalation of thyroid-stimulating hormone (MW: 27000 Da; TSH) were performed. Komada et al. (49) investigated the absorption of TSH solution and powder aerosol in rats with and without addition of an absorption enhancer following intratracheal administration. There was a more rapid absorption (no lag time after administration of the solution) and a higher bioavailability of TSH after administration of the solution than after administration of the powder aerosol. Changes of the pH value as well as addition of glycocholate were followed by an increased bioavailability of TSH after administration of the solution. In detail, bioavailabilities were 2.5, 16.2, 8.0, and 1.6% after administration of 40 mIU/kg by means of a pH 7.0 solution, a pH 7.0 solution with glycocholate, a pH 3.0 solution and dry powder in comparison with intravenous injection, respectively.

**Follicle-stimulating hormone**

Follicle-stimulating hormone (FSH) is a peptide hormone (MW: 36000 Da; FSH) which physiologically plays a role in the regulation of the menstrual cycle. The hormone is routinely administered by subcutaneous or intramuscular injection 8-10 days of each menstrual cycle for treatment of infertility (57-60). However, there are only few studies investigating the inhalant role in reproduction. Both consist of two heterodimeric peptides. The α-subunit is hormone-specific, hCG is essential for the establishment of pregnancy and fetal male sexual differentiation whereas LH induces sex hormone production and ovulation (62). Additionally, hCG in combination with luteinizing hormone agonists up to now plays a role in the treatment of cryptorchidism in males (63). In an early study, Komada et al. (49) investigated the absorption of hCG solution and powder aerosol in rats following intratracheal administration. Administration of the hCG solution was followed by a delayed increase of the plasma concentration. However, the observed lag time was more pronounced after administration of the powder aerosol than after administration of the solution. Changes of the pH value as well as addition of glycocholate were followed by an increased bioavailability of hCG after administration of the solution. In detail, bioavailabilities were 0.2, 4.8, 6.3, and 0.1% after administration of 2500 mIU/kg by means of a pH 7.0 solution, a pH 7.0 solution with glycocholate, a pH 3.0 solution, and dry powder in comparison with intravenous injection, respectively (49). Lombry et al. (64) were able to demonstrate that the low pulmonary absorption of hCG is caused by alveolar macrophages which serve as a primary barrier to pulmonary absorption. It was shown by these investigators that depletion of alveolar macrophages by liposome-encapsulated dichloromethylene diphosphonate was followed by a several-fold increase of hCG and IgG after intratracheal administration in rats.

**Luteinizing hormone-releasing hormone agonists and -antagonists**

A number of studies have been performed with luteinizing hormone-releasing hormone agonists (leuprolide; MW: 1200 Da; buserelin; MW: 1300 Da) which play a role in the treatment, e.g., of endometriosis, pubertas praecox, and prostate carcinoma and luteinizing hormone-releasing hormone antagonists (detirelix; MW: 1400 Da; cetorelix; MW: 1430 Da) which are used for treatment of infertility by intratracheal instillation or aerosol administration.

In an early study, Adjei et al. (65) investigated the effect of particle size on the bioavailability of leuprolide acetate in humans. In detail, solutions and suspensions of the nonapeptide were administered to 23 healthy males in a single-dose, four-period crossover study. Dependent on particle size and type of aerosol, the investigators observed an absolute bioavailability between 4 and 18% and a bioavailability corrected for the respirable fraction between 35 and 55%. Maximum plasma concentrations of leuprolide acetate were observed at about 1 to 2 h after inhalation also dependent on the aerosol formulation demonstrating a higher bioavailability of suspension aerosols compared with solution aerosol (65). In another study, the bioavailability of intratracheally administered leuprolide was investigated for a time period of 14 consecutive days in beagle dogs (66). Beside the pharmacokinetic parameters the investigators also studied the biological effect as determined by the plasma concentration of gonadotropins. The results demonstrated a linear dose-dependent increase in the pulmonary bioavailability of leuprolide acetate, with maximum concentrations about 30 min after administration, a possible bioavailability of 40% and a strong suppression of plasma gonadotropins with sequential increase in plasma leuprolide concentrations. However, the bioavailability of leuprolide at study day 1 was about double that on day 14 (66). The results, except the decrease of the bioavailability described in 1990, were confirmed in a similar study published in 1994 and the authors suggested that the observed decrease was caused by a mild inflammatory reaction of the lung tissue due to the use of the co-solvent alcohol in the first study (66, 67). The investigation of the impact of absorption enhancers and the type of administration was also subject of another study performed by
Adjei et al. (68) in rats and humans. In rats, the bioavailability after intranasal administration ranged between 8% and 46% and was significantly increased by addition of the absorption enhancers α-cyclodextrin and ethylene diamine tetraacetic acid (EDTA) showing an intraanimal variability of 30% to 60%. In contrast, the corresponding bioavailability in humans was only 2.4%, also showing significant intrapatient variability. The authors assumed the differences between rats and humans to be caused by a relevant loss into the mouth cavity and/or species-specific differences of the nasal mucosa. However, the inhalation delivery by means of a metered dose inhaler (MDI) in humans resulted in a slightly lower bioavailability and the mean values of the bioavailability of 28% and 6.6% (compared with subcutaneous administration) for suspension and solution aerosol, respectively, demonstrating the suitability of this method for drug administration (68). However, one should consider that taste masking excipients can negatively affect the performance of the formulations, e.g., by modification of the properties of the suspension itself and the alteration of propellant vaporization following actuation (69). More recently, Shahiwala and Misra (70) performed a pharmacokinetic study in rats by means of a liposomal leuprolide dry powder inhaler. Pharmacokinetics was determined by measurement of the plasma concentration of luteinizing hormone (LH). The authors observed a strong rise of the LH plasma concentration having a peak between 1 and 3 h after intratracheal administration and a half-life time of about 4.5 h resulting in a bioavailability of approximately 50% compared with the subcutaneous route of drug administration (70). Interestingly, we have found no publications regarding the inhalation of other luteinizing hormone-releasing agonists (e.g., buserelin and nafarelin), even though intranasal administration of these compounds is established in medical treatment (71).

Up to now, there are only a few studies dealing with the pulmonary administration of the decapeptides cetorelix (72-74) and detirelix (75, 76). A first, preliminary investigation of the pharmacokinetics of cetorelix was performed by Schreier et al. (74) who instilled the compound intratracheally to sheep. The investigators described a rapid absorption after intratracheal administration (t<sub>max</sub>) of 1.8 h, a half-life time (t<sub>1/2</sub>) of 11.6 h and a bioavailability of 15.4% (compared with intravenous administration). Another study was performed by Lizio et al. (72) who instilled different doses of cetorelix acetate intratracheally into rats and determined testosterone lowering effect and pharmacokinetics. Instillation of cetorelix was followed by a rapid increase of cetorelix plasma concentration and a rapid (within 1 to 2 h after administration) and long-lasting, dose-dependent decrease (up to 96 h after administration) of testosterone plasma concentration to subnormal levels. The corresponding pharmacokinetic data revealed a dose-dependent bioavailability of up to 76% compared with intravenous administration. Also, in the rat model a second study was performed by Lizio et al. (73) administering different formulations of a nebulized liquid aerosol. The authors investigated the pharmacokinetics of the inhaled compound by measurement of its serum concentration, the biological effect on the plasma testosterone concentration and the tolerability by means of histological evaluation of the lungs. Aerosol administration was followed by a reduction of the serum testosterone concentration to subnormal levels over a period of 24 h. The calculated mean values of bioavailability after intratracheal aerosol application, compared with the data obtained for intravenous administration, ranged from 37% to 77% dependent on the administered formulation. Furthermore, there were differences of the tolerability of the tested formulations ranging from locally intolerable to well tolerated (73). Bennett et al. (75) investigated the pharmacokinetics of the decapetide detirelix in dogs following intravenous administration, intratracheal instillation, and aerosol inhalation. They found a slow absorption (t<sub>max</sub> 6.5 h) of the compound and a relative bioavailability of 29% after instillation. Similar plasma profiles were found after aerosol administration. Repeated pulmonary administrations within an observation time of 5 months were not followed by a change of the pharmacokinetic pattern and a toxicological damage of the lung (75). In another investigation, Schreier et al. (76) studied the pharmacokinetics of detirelix administered by intratracheal instillation and aerosol inhalation in the unanesthetized sheep in comparison with intravenous administration. After instillation, the compound was rapidly absorbed showing a maximum of the plasma concentration 2 h after administration and a bioavailability of 10%. Reliable pharmacokinetic data after aerosol administration were obtained in one sheep only but demonstrated a similar profile.

**1-deamino-8-D-arginine vasopressin (dDAVP)**

The vasopressin analogue 1-deamino-8-D-arginine vasopressin (MW: 1100 Da; dDAVP) is used for treatment of enuresis where it is administered by means of nasal spray (77). However, few studies have investigated the feasibility of pulmonary dDAVP administration (78-80). In a first study, dDAVP beside bovine serum albumin and bovine IgG were used to assess the permeability of the respiratory tract after intratracheal instillation in young and adult rats. The investigators observed similar passage times into circulation of about 1 h in young and adult rats. However, the bioavailability was higher in young than in adults (45 vs. 20%) (78). In another study, Fulkesson et al. (79) used aerosols of the nonapeptide dDAVP and bovine serum albumin and studied the absorption of in rats of different age with and without lung injury. The investigators observed a similar time to peak of dDAVP of 0.5 to 1.0 h in adult rats (age: 100-120 d), a linear dose-response relation between the administered dose and the serum concentration and a bioavailability of dDAVP of 84%. However, the bioavailability of dDAVP was lower in younger rats. Inflammation of lung tissue resulted in an increase of the bioavailability of albumin and dDAVP; the increase was less pronounced for dDAVP than for albumin (79). The lung-to-blood passage of albumin and dDAVP was also determined after intratracheal instillation in young developing pigs (80). It was found that the plasma concentration of dDAVP reached a maximum 1 h to 3 h after instillation. However, the total lung passage of dDAVP decreased as a function of age (newborns: 75%, age 2 d: 44.1%, age 70 d: 23.6%), whereas no relevant age-dependent changes were observed for albumin (80).

**Erythropoietin**

Different types of this cytokine which serves as an erythropoietic growth factor (epoetin α: MW: 14 700 Da; epoetin β, γ, δ, ε, ω: MW: 18 200 Da) are used in clinical treatment of anemia to stimulate the erythropoiesis in the bone marrow, e.g., in patients with end-stage renal failure and cancer (8, 16, 81). However, due to its large molecular weight the pulmonary absorption without methods for absorption enhancement is low (8, 16, 82). Therefore, an interesting method based on Fc fusion proteins has been developed to improve pulmonary uptake and pharmacokinetics (16, 57-61, 83, 84). In a first study Bitonti et al. (83) investigated the pharmacokinetics in cynomolgus monkeys treated with an aerosol of two types of an erythropoietin Fc fusion protein (Epo-Fc monomer and Epo-Fc dimer) in comparison with normal erythropoietin administered via aerosol and intravenously. They observed a better absorption of the Epo-Fc
dimer when performing a shallow breathing pattern in comparison with a deep breathing pattern which was explained by a higher expression of the Fe receptor in the central airways. However, the bioavailability was only about 5% which was assumed to be caused by the molecular weight, steric hindrance between Epo and Fe mieties, charge or other physicochemical properties. In contrast, much higher bioavailabilities were obtained after inhalation of the Epo-Fc monomer and normal erythropoietin (35% and 15%, respectively). Interestingly, the bioavailability of the inhaled Epo-Fc monomer is very similar to that of normal erythropoietin after subcutaneous administration (83). Independent from the mode of administration (inhalation, intravenous administration) there were also differences of the plasma half-life time (t_{0.5}). In detail, values of t_{0.5} were 16, 25, 23, 6.5, and 6.3 h for the inhaled Epo-Fc dimer, the inhaled Epo-Fc monomer, the Epo-Fc monomer after intravenous administration, inhaled normal erythropoietin, and intravenously administered erythropoietin, respectively, demonstrating an increase of t_{0.5} due to the fusion to the Fe residual of the immunoglobulin. Functional analysis by measurement of reticulocytes in blood revealed that all types of inhaled erythropoietin and fusion proteins were biologically active, although there were differences in respect to the strength of the reticulocyte increasing effect (83). However, even though the results obtained in animals are promising, there are only few data of investigations performed in humans (57, 84). Different doses of the aerosolized Epo-Fc dimer (3, 10, and 30 µg/kg) were administered to healthy volunteers in a standardised breathing maneuver (70% central deposition, 30% peripheral deposition). The obtained results demonstrated dose-dependent serum concentrations of Epo-Fc (C_{max}; 0.2, 1.2, and 7.1 ng/ml, data for doses of 3, 10, and 30 µg/kg, respectively) and serum concentration vs. time curves (AUC; not available, 42.1, and 271.1 ng h ml^{-1}, respectively), whereas there were no differences of the time of the maximum serum concentration (t_{max}; 21.4, 18.0, and 22.5 h, respectively) and the terminal half-life time (t_{1/2}; not available, 14.4 and 15.7 h, respectively). Additionally, the best biological response (i.e., increase of circulating reticulocytes in blood) was observed in the group receiving the highest dose (57, 58, 84).

Granulocyte-monocyte colony-stimulating factor (GM-CSF)

Treatment with recombinant granulocyte-monocyte colony-stimulating factor (GM-CSF; MW: 14600 Da) has been approved for therapy of patients to recover neutrophils from induction chemotherapy for acute myelogenous leukemia, mobilization and following transplantation of autologous peripheral progenitor cells, myeloid reconstitution after allogeneic bone marrow transplantation, and bone marrow transplantation failure or engraftment delay (81). The glycoprotein GM-CSF promotes maturation of bone marrow cells into monocytes and dendritic cells, but also affects functional activities of mature effector cells involved in antigen presentation and cell mediated immunity (85). For this purpose, the cytokine is usually administered by means of subcutaneous injection or infusion (4). However, several other potential clinical indications (immune modulation in infectious diseases, use as a vaccine adjuvant, use in antitumor therapy, treatment of mucositis, stomatitis and diarrhea, use for improvement of wound healing, treatment of alveolar proteinosis) have been investigated and in some of them GM-CSF had been administered by means of aerosol inhalation (81, 85). Two of these clinical indications, use in hematopoiesis restoration and treatment of alveolar proteinosis, are discussed below in more detail as aerosols are used for these therapies. Interestingly, aerosolization by means of air-jet nebulisers does not affect the stability of the cytokine (4).

Feasibility of aerosol administration of GM-CSF was subject of an early study performed in cynomolgus monkeys more than 15 years ago (86). The animals were treated with GM-CSF by continuous infusion for 2 weeks or aerosol inhalation for 1 and 2 consecutive days, respectively; treatment with bovine serum albumin served for control. The investigators observed a strong dose-dependent increase of the number of neutrophils and macrophages in bronchoalveolar lavage, augmented respiratory burst, and increased capacity to bind and ingest Staphylococcus aureus in animals treated with aerosolized GM-CSF, whereas only minor changes were found after continuous infusion. Intravenously administered GM-CSF and aerosolized GM-CSF caused an increase of the number of circulating myeloid cells (predominantly neutrophils) in peripheral blood demonstrating the systemic effect even after aerosol inhalation. The increase was higher after aerosol administration for 2 days than for 1 day and was most marked 3 days after aerosol exposure. However, despite the strong effect of aerosolized GM-CSF on pulmonary phagocytic cells the authors observed an increase of only some biochemical parameters in BAL serving as an indicator for potential lung injury (86).

A larger number of studies investigated the effect of GM-CSF on pulmonary alveolar proteinosis (87) and in humans (88-95). In brief, pulmonary alveolar proteinosis is an orphan disease, with less than 500 reported cases until 2006, which was firstly described in 1958 by Rosen et al. (96). It may be the first human disease by which an autoantibody against a growth factor binding to receptors present on hematopoietic cells and alveolar macrophages is linked to disease pathogenesis. In an early study, Reed et al. (87) administered GM-CSF to GM-CSF deficient mice by means of aerosol and intraperitoneal injection. Aerosol administration of GM-CSF was followed by a return of lung histology, alveolar macrophage differentiation, and surfactant protein B immunostaining toward normal levels and by a significant decrease of alveolar and lung tissue saturated phosphatidylcholine and surfactant protein B concentrations, whereas cessation of aerosol therapy resulted in increased saturated phosphatidylcholine pool sizes returning to pretreatment levels. In contrast, no such improvement of pulmonary alveolar proteinosis was observed after intraperitoneal administration of GM-CSF. In the following years, beginning with the case report published by Seymour et al. (90), a number of studies have demonstrated that inhalant administration of GM-CSF aerosol alone, or if necessary in combination with whole lung lavage, is a save and efficient therapy for treatment of pulmonary alveolar proteinosis even for a longer treatment period (89-95).

Other studies investigated the effect of inhaled recombinant GM-CSF on different types of cancer (97-101). A first study investigating the effect of aerosolized GM-CSF was initiated by Anderson et al. (97). These authors studied 7 patients with lung metastases of leiomyosarcoma, renal cell carcinoma, Ewing’s sarcoma, osteosarcoma, and melanoma. The patients were treated with an aerosol of yeast-derived glycosylated recombinant GM-CSF. After performing a dose escalation study with three different doses from 60 µg/dose BID for 7 days up to 240 µg/dose BID for 7 days the authors settled an intermittent aerosol therapy in 6 patients; one patient was excluded due to medical reasons and poor compliance prior to dose escalation. There were no relevant changes of lung function and no side effects within the high dose observation period. Parameters of the blood cell count revealed only minor increases of white blood cells and neutrophils under therapy which failed to get significant. Treatment with aerosolized GM-CSF resulted in a complete response in one patient, a partial response in one more patient, and a stabilization of the pulmonary metastases up to 6 months in three patients. However, one patient’s lung metastases were progressive (97). Based on these
findings a similar study was performed later by Rao et al. (101) in 45 patients with metastases of different types of cancer (predominantly melanoma: 14, renal cell carcinoma: 12, osteogenic sarcoma: 7, other tumors (mostly other sarcoma): 12) treated with 250 μg/dose twice a day using an one week on, one week off schedule and the same recombinant GM-CSF. In total, 24 of the patients (8 of 13 with sarcoma, 6 of 14 with melanoma, 5 of 12 with renal cell carcinoma) had a disease stabilization or partial regression lasting for a mean time of 10 months. The most frequent cause of treatment discontinuation was progression of the disease. However, even though toxicities reported by the patients were mostly self-limiting (18 cases) in one patient with severe chronic obstructive pulmonary disease (COPD) treatment had to be discontinued due to adverse effects (101). Recently, Markovic et al. (100) published the results of a dose escalation study performed in 40 patients with pulmonary metastases of melanoma in which aerosolized GM-CSF (sargramostim) was administered in doses up to 2000 μg twice/day. Escalating doses were administered until observation of disease progression or severe toxicity to find a dose where the majority of treated patients developed an antitumor immunity. 34 of the patients were followed to death or a minimum of 14 months; the others declined participation prior to treatment, were ineligible to participate due to a low FEV1 or died directly after study had begun. The investigators reported an acceptable toxicity in the treated patients. Greatest changes of antitumor immunity were achieved at highest doses of inhaled GM-CSF. Times of progression-free survival were 4 - >19.5 months (median: 8.4 months), 1 - 15.8 months (median: 2 months) and 1 - 4.6 months (median: 1 month) in the 5 patients who developed an immune response, the 20 patients without immune response, and the 9 patients without immune data, respectively. There is also an additional case report of a patient with the Sezary syndrome, the leukemic and most aggressive subtype of cutaneous T-cell lymphoma with poor prognosis, sufficiently treated by means of a combination of extracorporeal photophoresis and inhalation of aerosolized GM-CSF (sargramostim) (99).

Granulocyte colony-stimulating factor (G-CSF)

Another cytokine, granulocyte colony-stimulating factor (G-CSF; MW: 18800 Da) has been approved for treatment of febrile neutropenia from chemotherapy for non-myeloid malignancies. In clinical routine, it is administered by means of subcutaneous injection or infusion and promotes maturation of bone marrow cells into granulocytes (4, 102). A number of experimental studies in animals addressed the administration of G-CSF by means of an aerosol. However, there is lack of human studies in this field (81). Possibly, this is the consequence of the instability of the glycoprotein in air-jet nebulisers (4). A first study was performed by Niven et al. (103) who administered recombinant methionyl human G-CSF to hamsters by means of intratracheal instillation, subcutaneous injection, and intracardiac injection. Dose response curves of the white blood cell count were determined for all routes of G-CSF administration. The investigators found a rapid increase of the plasma concentration (about 20% of the absorbable dose 6 min after administration) reaching a maximum serum concentration (Cmax) within 1 and 2 h after intratracheal instillation. Values of the bioavailability were 45.9% and 62% for the administered dose and the dose reaching the lung lobes, respectively. The slope of the terminal phase after intratracheal administration and the corresponding half-life time (t1/2) were not different to those after intracardiac administration (t1/2: 2.6 vs. 2.9 h) indicating similar clearing kinetics of G-CSF. All routes of drug administration caused a similar increase of white blood cells in peripheral blood, demonstrating the functional activity of the absorbed compound. Additionally, there was an increase of neutrophils in bronchoalveolar lavage fluid (BAL) which, however, was not related to an inflammatory response (103). In another study, resorption and biological activity of different doses of two G-CSF powder formulations and variant doses of G-CSF solution (5 and 500 μg/kg) were investigated in rabbits after tracheal insufflation and instillation, respectively. Administration by means of subcutaneous and intravenous injection served for reference. Parameters of pharmacokinetics and pharmacodynamics were determined for all doses and routes of drug administration accordingly (104). Plasma concentration vs. time profiles for intratracheal insufflation and instillation showed no differences (tmax: 1 h-2 h), but the obtained values for tmax were much shorter than those after subcutaneous administration (tmax: 6 h-10 h, dose dependent). Insufflated powders were less efficiently dosed to the lung lobes than instillates (14.7±10.5% vs. 60.1±10.6%) resulting in a dose-dependent bioavailability ranging from 4.9 to 32.9%. However, after subcutaneous administration, the bioavailability values of 11.0±7.9 and 95.3±7.9% were observed for low and high doses, respectively. Independent from the route of its administration, G-CSF induced an increase of the white blood cell counts in peripheral blood. However, there were differences in the response depending on the route of administration and the given dose. In detail, the strongest increases of the white blood cell counts were observed 20-50 h after subcutaneous and intravenous administration of 500 μg/kg, respectively, whereas intratracheal instillation and insufflation were followed by a lower and even shorter biological response. Comparable results were obtained after administration of 5 μg/kg; however, the intensity and duration of the biological effect were smaller (104). Niven et al. (105) also investigated pharmacokinetics and pharmacodynamics of aerosolized and intratracheally instilled G-CSF and two preparations of monPEGylated G-CSF (PEG of 6000 Da and 12000 Da, respectively) in rats. Based on the results of prior investigations all protein preparations were stabilized by addition of Tween80 (4). Strong differences depending on the type of the administered protein (PEGylated vs. non-PEGylated) and the mode of administration (aerosol inhalation vs. tracheal instillation) were observed. In detail, there was a better absorption for the aerosol than for the instillate and the non-PEGylated protein was better absorbed than the PEGylated protein. Corresponding data of tmax and Cmax were 21.7±4.8, 168±31, 100±17, and 310±121 min, and 598±135, 182±14, 105±12, and 65.9±14 ng/ml for aerosolized G-CSF, aerosolized PEGylated G-CSF (12000 Da), instilled G-CSF, and instilled PEGylated G-CSF (12000 Da), respectively. Accordingly, the values of the relative bioavailability were 65.9±14, 12.3±1.9, 12.0±1.5, and 1.6±0.1% for aerosolized G-CSF, aerosolized PEGylated G-CSF (12000 Da), instilled G-CSF, and instilled PEGylated G-CSF (12000 Da), respectively. However, the white blood cell count demonstrated no differences regarding to the type of the administered protein and the mode of administration (105). The pulmonary absorption of G-CSF was also the subject of two studies performed in rats by Machida et al. (106, 107). In the first study, the investigators studied the pharmacodynamics and the biological effect of different doses of recombinant human G-CSF after intratracheal instillation and intravenous and subcutaneous injection, respectively. It was found that intratracheal instillation of 100 μg/kg was followed by the bioavailability of 11.6% and 27.4% compared with intravenous and subcutaneous injections which were about 10-times higher than the corresponding results after intranasal administration published before (108). Administration of increasing G-CSF doses resulted in a dose-dependent increase of total leukocyte numbers depending on the mode of administration. In case of intratracheal administration of G-CSF, the peak number of leukocytes in blood was observed 8 h after administration;
however, administration of higher doses (up to 200 µg) were followed by higher leukocyte numbers at a later time after administration (106). In the later study, the same authors investigated the effect of various absorption enhancers. It was found that co-administration of the surfactants Laureth-9 and sodium glycocholate, and the protease inhibitors p-amidinophenyl methane sulfonyl fluoride HCl (p-APMSF, serine protease inhibitor), aprotinin (trypsin inhibitor), and bestatin (leucine protease inhibitor) was followed by a strong improvement of the bioavailability of G-CSF after intratracheal administration compared with the intravenous and subcutaneous administration route, suggesting that the low bioavailability of G-CSF is caused by both, a low membrane penetration and a proteolytic degradation (107).

Safety of the inhalation of peptides and proteins

Analysis of safety and tolerability of pulmonary administered compounds firstly includes the investigation of their activity after inhalation, which can be largely different compared with subcutaneous administration. For instance, inhaled insulin causes a more rapid decrease of the blood glucose concentration than subcutaneously administered insulin (1, 8, 11, 12, 17, 25, 26). Furthermore, pulmonary diseases may complicate or prevent inhalant drug therapy under some circumstances (8, 11, 17). Additionally, an incompatibility of the inhaled pharmaceutical can be due to the active compound itself as well as other compounds added to improve stability and pulmonary absorption of biomolecules (1, 16). For instance, inhaled peptides and proteins can cause an immunization (8, 10, 11, 17, 26), but also can have specific effects on the target organ lung (e.g., growth stimulating effect of insulin) (12, 17, 26) and a chronic administration of absorption enhancers (e.g., alcohol, bile acids, and cyclodextrins) can damage alveolar epithelium (15, 16, 82). Last not least, administration of compounds by means of microparticles and liposomes can harm the lung by different mechanisms (1, 15).

As it is demonstrated in this review, data for systemic treatment by inhalation are available for a large number of biomolecules. However, these data are predominantly based on the results of animal studies. Additionally, only for a very small subset of biomolecules (insulin, heparin) data for the long-term tolerance have been published and only insulin received market approval, but failed market acceptance (1, 8, 11, 12, 16, 17, 20-26). Nevertheless, the experimental data on inhalant drug therapy of systemic diseases are often promising. Therefore, future studies should be performed to get a novel type of therapy which is safe and convenient for the patients.

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