Peptides and Proteins: Pulmonary Absorption

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INTRODUCTION
Inhalation of drugs is very efficacious for the treatment of lung diseases and a continuously increasing number of inhaled drugs are becoming available on the market. Inhalation of drugs allows a targeted therapy with high drug concentrations in the tissue of interest, low systemic drug exposure, and thereby reduced systemic side effects. In addition, it allows a rapid onset of therapeutic action and it is a convenient mode of drug delivery.

Inhalation may also be an optimal route for the systemic administration of drugs because pulmonary drug absorption is high and rapid and first-pass hepatic metabolism is avoided. Peptides and proteins are more efficiently absorbed from the lungs than from any other noninvasive route of drug administration. For instance, insulin can absorb from the lungs with a bioavailability of 30% relative to subcutaneous injection, while it reaches at most 1% following oral, sublingual, nasal, or transdermal administration without chemical enhancer (1,2). These absorption features originate from the large absorptive surface area of the alveoli, the very thin diffusion path to the bloodstream as well as the local high blood flow. Yet, administration of drugs to the healthy lungs can raise toxicity issues in the long term, and it would be wise to consider it for the treatment of short course diseases. MAP Pharmaceuticals currently seeks FDA approval for Levadex®, a dihydroergotamine inhalation aerosol for the treatment of migraine, in line with this idea (3).

This chapter provides information about the advances in pulmonary delivery of peptides and proteins used in both local and systemic therapy. Inhaled peptides and proteins that are currently undergoing various phases of clinical trials are presented. The different biological pathways that these molecules can follow after deposition in the lung are described, including pulmonary absorption to the bloodstream. Furthermore, the available in vitro and in vivo models for the assessment of pulmonary absorption of peptides and proteins are outlined. The impact of smoking and various pulmonary disease conditions on the pulmonary fate of inhaled peptides and proteins is shown. Finally, a discussion about stability issues that arise during their formulation, storage, and aerosolization is given.

LUNG PHYSIOLOGY
The respiratory system resembles an inverted tree where the trachea divides into two main bronchi. Each bronchus further subdivides into progressively smaller bronchioles until it reaches the smallest airspaces called alveoli. The lung consists of two functional spaces: the conducting zone (16 first generations) and the respiratory zone (7 last generations). In the conducting region, the air is filtered, warmed, and humidified, whereas in the respiratory region, gas exchange between airspaces and blood capillaries occurs. The airway bifurcations become smaller in diameter and longer but higher in number and larger in total cross-sectional area. Consequently, the alveoli provide a total surface area that reaches 100 m², which is substantially larger compared to the 0.25 m² surface area of the airways (4).

Two different epithelia line the conducting and respiratory zones (Fig. 1) (5,6). A pseudostratified columnar epithelium lines the proximal conducting airways and is composed of ciliated columnar cells, goblet or mucosecreting cells, and basal or progenitor cells (7). It is progressively replaced by a simple cuboidal cell layer in the more distal airways by a very thin epithelial lining in the alveoli. Squamous type I pneumocytes cover 95% of the alveolar surface, owing to their large apical surface and thinness (0.05 µm). Cuboidal type II pneumocytes are located in the corners of the alveoli. They produce the lung surfactant and are progenitor for type I cells.

Mucociliary clearance is one of the most important defense mechanisms to eliminate dust and microorganisms in the lungs (8). The mucus is produced by goblet cells and sub-mucosa glands and protects the underlying mucosa from dehydration. It covers the entire airway surface and its thickness ranges from 5 µm to 55 µm. It consists of an upper gel phase made of 95% water, 2% mucin (a highly glycosylated and entangled protein) as well as salts, proteins, and lipids (9). A periciliary liquid layer underlies the mucus gel and its low viscosity allows effective cilia beating. The mucus is transported by the coordinated beating of the cilia and by expiratory airflow toward the oropharynx. Mucus, cells, and debris coming from the nasal cavities and from the lungs meet in the pharynx, are mixed with saliva, and are swallowed. Mucus velocity slows down when descending the respiratory tree, with 3 orders of magnitude faster mucus velocity at generation 0 as compared to generation 16. This counterbalances the high number of peripheral airways and thereby the accumulating amounts of mucus to be cleared by the central airways and trachea (10).

Pulmonary surfactant is responsible for biophysical stabilizing activities and innate defense mechanisms. It lines the alveolar epithelial surfaces and overflows into the conductive
airways so that the surfactant film is continuous between alveoli and central airways (11). Pulmonary surfactant is composed of 80% phospholipids, 5–10% neutral lipids (mainly cholesterol), 5–6% specific surfactant proteins, and 3–4% nonspecific proteins (12). The phospholipids are mainly responsible for forming the surface-active film at the respiratory air–liquid interface. Half of surfactant phospholipids by mass are composed of disaturated species, mainly dipalmitoylphosphatidylcholine. Specific surfactant proteins include SP-A, SP-B, SP-C, and SP-D. SP-A and SP-D are hydrophilic, whereas SP-B and SP-C are hydrophobic. SP-A is able to bind multiple ligands, including ligands on the surface of pathogens. SP-A recognition by specific receptors on alveolar macrophages stimulates phagocytosis. SP-B is strictly required for the biogenesis of pulmonary surfactant. Both SP-B and SP-C promote rapid transfer of phospholipids into air–liquid interfaces.

Luminal airway and alveolar macrophages are at the forefront of lung defense, and their primary role is to participate in innate immune responses, that is, chemotaxis, phagocytosis, and microbial killing (13). They also down-regulate adaptive immune responses and protect the lungs from T-cell-mediated inflammation (14). Alveolar macrophages are tightly applied on the surface of respiratory epithelia. They are immersed in the lung lining fluid beneath the surfactant film. Although they occupy only 1% of the alveolar surface, they are capable of cleaning particles from the entire alveolar surface due to amoeboid movements (13). In contrast to surface macrophages, interstitial macrophages are primarily involved in adaptive immunity by interacting with lymphocytes through antigen presentation and production of cytokines (13).

The lung presents a lower level of metabolism than the gastrointestinal tract and liver. Yet, various peptidases are distributed on the surface of different cell types in the lung, including bronchial and alveolar epithelial cells, submucosal glands, smooth muscles, endothelial cells, and connective tissue. Proteases are largely present in lysosomes (15). Proteases that degrade the extracellular matrix are secreted by different structural cells, or membrane bound (16). Proteases play an essential role in cell and tissue growth, differentiation, repair, remodeling, cell migration, and peptide-mediated inflammation (17). Proteases can also be released in the airspaces by activated macrophages and neutrophils in case of inflammation in the respiratory tract (18).

Blood supply to the lungs is divided among the pulmonary and systemic circulations (19). The pulmonary circulation consists of the pulmonary artery that leaves the right heart, branches into a dense pulmonary capillary bed that surrounds the alveoli, and finally coalesces into the pulmonary vein that drains into the left heart. One hundred percent of the cardiac output flows through the pulmonary circulation. Its principal functions are gas exchange with air in the alveoli and nutrients supply to terminal respiratory units. The lungs receive a second blood supply through the systemic circulation, commonly referred to as the bronchial circulation. The bronchial circulation originates from the aorta and provides oxygenated blood and nutrients to all structures of the tracheobronchial tree. Lymphatic vessels exist in close proximity of major blood vessels and airways (20).

DEVELOPMENT STATUS OF INHALED PEPTIDES AND PROTEINS

Although the efforts performed in the field of pulmonary delivery of peptides and proteins have been tremendous, there are still a very limited number of inhaled macromolecules available on the market (Table 1). Yet, there are a growing number of inhaled peptides and proteins undergoing various phases of clinical trials, those developed for local therapy being the most promising.
The first inhaled insulin product for the treatment of patients with type 1 and type 2 diabetes mellitus was approved under the name Exubera® in January 2006. However, less than 2 years later, the drug was withdrawn from the market due to disappointing sales. Prescriptions amounted to less than 1% of the insulin market because the dry powder inhaler failed to gain acceptance of patients and physicians. Exubera® did not present improved efficacy as compared to short-acting subcutaneous insulins (Fig. 2). The time to maximum serum insulin concentrations was similar following inhalation using Exubera® and following injection of rapid-acting insulin analogs (22). Insulin bioavailability using Exubera® was approximately 10% relative to subcutaneous regular human insulin. Exubera® marginally decreased patients’ breathing ability, and regulators required patients to take lung function tests before and during treatment, which increased cost and inconvenience (23).

The large size of the Exubera® inhaler was also an issue. Another inhaled insulin product, AFREZZA™, is currently under review by the Food and Drug Administration (FDA) for use in patients with diabetes (24). AFREZZA™ is an ultra-rapid acting insulin comprising Technosphere® insulin powder in unit-dose cartridges for administration with the inhaler. The Technosphere® powder formulation is prepared by precipitating insulin from solution onto preformed diketopiperazine particles, which readily dissolve once in the lung environment. AFREZZA™ appears to overcome several limitations of Exubera®. Technosphere® insulin is both rapidly absorbed and eliminated, and its pharmacokinetic profile mimics more closely normal physiologic insulin release than injection of regular human insulin and of rapid-acting analogs. Insulin bioavailability using AFREZZA™ reaches 30% relative to subcutaneous regular human insulin. The inhaler is small and discrete. AFREZZA™ has demonstrated a favorable safety and tolerability profile in clinical studies. However, a small reduction in pulmonary function also appeared in patients who received Technosphere® insulin (1,25).

The development of inhaled insulin began in 1990 and led to the investigation of the pulmonary administration of many other systemically acting therapeutic peptides and proteins. Preclinical studies have been numerous but only a few small-scale clinical trials have been conducted. These included studies on LHRH analogs (27), salmon calcitonin (28), human growth hormone (hGH) (29), and an erythropoietin-Fc fusion protein (30). Yet, following withdrawal of Exubera® from the market, these clinical trials have not been pursued. Only one pharmaceutical company (MannKind corporation) currently pursues the development of inhaled peptides for a
systemic action, these include insulin (AFFREZA™) and glucagon-like peptide 1 (GLP-1). Pulmonary administration of GLP-1 adsorbed on Technosphere microparticles is undergoing initial clinical investigation (31). The pulsatile administration of GLP-1 through the lungs is beneficial as the gastrointestinal intolerance observed after subcutaneous injection is avoided. This drug may be used alone or in combination with prandial insulin in patients with type 2 diabetes.

While FDA approval of new molecular entities has steadily decreased over the last 15 years (from 53 approvals in 1996 to 15 in 2010), FDA approval of biomolecules has remained constant with an average of four to five approvals per year (32). Therefore, the proportion of biomolecules delivered by inhalation is also expected to grow. A decoy form of IL-8 can potentially treat chronic obstructive pulmonary disease (COPD) following pulmonary administration (33). COPD is characterized by a neutrophilic inflammation of the airways where IL-8, a major chemokine, plays a central role. This decoy protein is an engineered version of human IL-8, with higher affinity for glycosaminoglycans present on endothelium cell surfaces and in which the neutrophil-binding domain has been removed. Therefore, it acts as an anti-IL-8 product. This protein is currently in phase I study which started in early 2012. A single-variable domain of a camelid immunoglobulin, called a nanobody®, has been used for the binding of the respiratory syncytial virus fusion protein and thereby for virus neutralization (34). This candidate drug entered Phase I clinical trials in December 2011. Nanobodies® appear suitable for inhalation as they are very stable with a low propensity to aggregate. It is also noteworthy that they can be manufactured at relatively low cost in microbial systems.

FATE OF PEPTIDES AND PROTEINS IN THE LUNGS

The fate of drugs following inhalation depends on their site of deposition within the lungs. Aerosol particles deposited in the tracheobronchial tree come into contact with the mucus, and the peptide or protein transported within the particle dissolves within the mucus. The macromolecule can then be cleared by the mucociliary escalator into the gastrointestinal tract or can diffuse in the mucus and cross the airway epithelium. On the other hand, particles deposited in the alveolar region initially come into contact with the thin layer of lining fluid coating the alveolar epithelium. The dissolved peptide or protein can then be subjected to clearance by alveolar macrophages or can be transported across the alveolar epithelium into the bloodstream. The lungs exhibit a decreased proteolytic activity compared to the gut, enzymes are present though. Peptides and proteins may be enzymatically degraded either extracellularly (by membrane-associated proteases and peptidases) and/or intracellularly (within macrophages and epithelial cells) (35).

Deposition in the Respiratory Tract

Deposition is the process that determines the fraction of the inhaled particles that will be caught in the respiratory tract and will not be exhaled. The aerodynamic diameter, \( d_{\text{aer}} \), of an inhaled particle has a major impact on its site of deposition within the lungs. The \( d_{\text{aer}} \) can be conceptualized as the diameter of a spherical particle with a density of 1 g/cm³ \( (\rho_0) \), such as a water droplet, which has the same velocity as the particle of interest in still air. It is defined by the equation:

\[
d_{\text{aer}} = \frac{d}{\sqrt{\frac{\rho}{\rho_0 \chi}}}
\]

where \( d \) is the geometric diameter of the particle, \( \rho \) is the particle density, and \( \chi \) is the particle dynamic shape factor denoting deviation of shape from sphericity (36).

Pulmonary deposition of particles occurs mainly by three mechanisms: inertial impaction, gravitational settling, and Brownian diffusion. It depends on aerodynamic particle size, on inhalation flow, and on lung anatomy. Large particles \( (d_{\text{aer}} > 5 \mu m) \) deposit in upper airways (mouth,
trachea, and main bronchi) by inertial impaction where the airstream velocity is maximum. Inertia refers to the inability of inhaled particles to follow the changes in direction and speed of the inspired airflow within the respiratory tract. Therefore, particles retain their original direction, “crashing” on the airway wall. Smaller particles (\(d_{\text{ave}} = 1\) to 5 \(\mu\)m) usually pass through the larger airways and reach the deep lung (lower airways and respiratory bronchioles), where they deposit by gravitational settling. In this region, the airstream velocity markedly decreases due to the dramatic increase in total airway cross-sectional area. Therefore, the particles “fall” on the airway wall because of gravity. Very small particles (\(d_{\text{ave}} < 1\) \(\mu\)m) remain suspended in the air and up to 80% of the inhaled bolus can be exhaled due to low inertia and low sedimentation.

The effectiveness of inertial impaction and sedimentation varies with the breathing pattern and with the anatomy of the respiratory tract. Slow inhalation is generally preferred to minimize inertial impaction in upper airways and to increase penetration into the lungs. A breath hold gives time to particles that have penetrated deep into the lungs to sediment on airway surfaces. Variations in airways anatomy between individuals, that is, airway dimensions and branching angles, lead to variations in aerosol deposition between subjects. In patients with asthma, COPD and cystic fibrosis, there is a systematic variability in airway anatomy, because the pulmonary airways may be narrowed by a combination of bronchospasm, inflammation, and mucus hypersecretion. Airway narrowing increases the likelihood of deposition by impaction, as well as creates turbulent airflow in regions of the lungs where airflow would otherwise be laminar. Aerosol deposition in central airways may therefore occur more readily in patients than in healthy subjects, and peripheral airway deposition may consequently be lower (37).

Drug delivery inhalers can be divided into three different categories: nebulizers, metered-dose inhalers, and dry powder inhalers. Therapeutic peptides and proteins have been delivered to the lungs using nebulizers and dry powder inhalers. The drug must reach the target receptors in an adequate amount to effectively treat the disease in focus. Medical inhalers generate particles with \(d_{\text{ave}}\) in the micron-size range for both local and systemic treatment. Particles with a \(d_{\text{ave}}\) between 3 \(\mu\)m and 10 \(\mu\)m are used for deposition in the tracheobronchial tree to treat the airways; whereas particles with a \(d_{\text{ave}}\) between 1 and 3 \(\mu\)m are used for deposition in the alveolar region for systemic drug absorption.

Conventional inhalers typically deliver 10% of their nominal doses to the lungs and lung deposition generally increases with peak inspiratory flow rate (38). New technology inhalers have largely improved these features. For instance, the AIR dry powder pulmonary system reaches a lung deposition of the nominal doses of 50%, and lung deposition does not depend on peak inspiratory flow rate (38). An AERx prototype high technology nebulizer was capable of delivering 80% of the nominal dose to the lungs (39). The MedTone dry powder inhaler, used to deliver Technosphere insulin, reaches a lung deposition of 40% of the initial cartridge load (40).

Interaction with the Air–Liquid Interface
Following pulmonary deposition, macromolecules interact with the air–liquid interface. The large surface area of the lungs is favorable to adsorption of proteins at the air–liquid interface, to protein unfolding and aggregation (41–43). Proteins can also bind endogenous components in the lung lining fluid and form agglomerates. Protein aggregates are likely to be scavenged by alveolar macrophages and be degraded. For instance, hGH has been shown to aggregate in the lungs following intratracheal instillation in adult rats (42). After deposition in the alveoli, hGH was concentrated in a thin layer at the air-epithelial boundary, little hGH penetrated respiratory epithelia, and the protein was largely taken up by alveolar macrophages (42,43). Aggregates of hGH were visible in a gel filtration chromatogram carried out on lung homogenates (42).

Soluble proteins may minimally perturb surface-active lipid films, with minor reorganization at low concentrations of the protein (44). Interactions of serum and serum proteins with pulmonary surfactant have been largely investigated in vitro because serum leakage into the alveolar space has been assumed to be the primary cause of surfactant dysfunction in acute respiratory distress syndrome. Air interface adsorbed films of bovine lipid extract surfactant could not attain equilibrium surface tension value in vitro, in a tensiometer, when bovine serum albumin was added to the surfactant. Albumin itself being surface active adsorbed at the air–liquid interface and inhibited the surface adsorption of the lipid extract surfactant. Yet, these effects were observed at high albumin to surfactant relative concentrations (1:1 w/w), concentrations which are well above the concentrations of therapeutic proteins attained locally following pulmonary delivery.

Mucociliary Clearance
Mucociliary clearance clears proteins that stick to mucin fibers or freely diffuse in the mucus but are unable to cross the airway epithelium. Mucin forms a network with a mesh spacing between 20 nm and 800 nm, which is much larger than the hydrodynamic diameter of most globular proteins (2–15 nm) (45). Yet, some proteins can make low-affinity bonds with the mucin and their diffusion can be hindered in mucus. Adhesion can involve electrostatic interactions with the carboxyl or sulfate groups on the mucin, low-affinity bonds with hydrophobic domains and hydrogen bonds.

Olmsted et al. studied the diffusion of macromolecules in human cervical mucus. Nearly every soluble globular protein investigated diffused in mucus as fast as it diffused in PBS (\(D_{\text{mucus}}/D_{\text{PBS}} = 1\) (45–47). However, polyvalent antibodies were retarded in mucus due to low-affinity bonds of the Fc domains with mucin fibers (45). This was concluded after a comparative study of the diffusion rate in mucus of full-length IgM and IgM after removal of Fab regions, that is, a pentameric ring of Fcs joined by the IgM j-chain. Both proteins were identically slowed in mucus. The binding between antibodies and mucin must have very low affinity because the diffusion of IgG, with only one Fc region, was not slowed significantly in mucus but antibodies...
with multiple Fcs as IgM and small aggregates of IgA were significantly slowed (Fig. 3). This suggests that antibodies accumulating on the surface of a pathogen may be able to form a sufficient number of low-affinity bonds to trap the pathogen in the mucus.

Lay et al. have monitored the retention and clearance of radiolabeled human serum albumin and radiolabeled sulfur colloid (220 nm insoluble particles) following localized deposition in a bronchus in dogs (48). Both compounds were cleared by mucociliary clearance but albumin was cleared more slowly than sulfur colloid. This indicates that a low-permeating water-soluble material as albumin remains in contact with the airway epithelium to a greater extent than does a solid insoluble particle. Albumin likely diffused to a greater extent than sulfur colloid into the peri-ciliary sol layer, which is transported less efficiently than the mucus gel layer during mucociliary clearance.

**Alveolar Macrophages**

Alveolar macrophages are a primary barrier to the transport of large proteins from the airway lumen into the bloodstream (41). Lombray et al. showed that depletion of alveolar macrophages by liposome-encapsulated dichloromethylene diphosphonate (Cl₂MDP) caused severalfold enhancement in systemic absorption of immunoglobulin G (150 kDa) and human chorionic gonadotropin (39.5 kDa) following intratracheal instillation in rats (Fig. 4). Large proteins are slowly transported across the alveolo-capillary barrier and can remain within the airspaces for several hours. This gives time to alveolar macrophages to engulf them by pinocytosis or “cell drinking,” the uptake of fluids and soluble compounds. In contrast to large proteins, no increase in pulmonary absorption of the peptide insulin (5.8 kDa) and of the small protein hGH (22 kDa) was associated with the depletion of alveolar macrophages (49). Insulin and growth hormone remained in the airspaces for less than 1 hr in rats, indicating that these compounds crossed the alveolar epithelium quickly, presumably preventing major uptake and degradation by alveolar macrophages.

It should be noted that a large molecular weight does not systematically involve a long residence time within the airspaces and consequently an uptake by alveolar macrophages. Other mechanisms can affect the rate of transport of proteins through the epithelium relative to the rate of alveolar macrophages uptake. For instance, there is evidence that for certain endogenous molecules that normally occur in lung lining fluids, for example, albumin, immunoglobulins, and transferrin, there are specific receptor-mediated transport mechanisms on the alveolar epithelial cell that enable these proteins to be absorbed at higher rates than expected (50). In addition, the rate of endocytosis by alveolar macrophages can be affected by the physicochemical properties of the proteins, with hydrophobicity and a global cationic charge increasing adsorptive endocytosis of proteins (49).

**Peptidase and Protease Activity**

Although the lungs are a far less hostile metabolic environment than the gastrointestinal tract, proteolytic enzymes are still present (51). Enzymatic degradation of inhaled proteins and peptides may occur prior or along their transport across the lung epithelium. Baginski et al. analyzed the mRNA expression of proteolytic enzymes in cell lines and primary cells of the human respiratory epithelium (52). They focused their investigation on secreted and membrane-bound peptidases. Many enzymes were shown to be expressed in human respiratory epithelial cells, but at different
levels according to the cell type. All respiratory cells expressed a smaller number of peptidases than Caco-2 cells, an intestinal epithelial cell line.

Small peptides are prone to degradation by peptidases located at the apical surface of the airway and alveolar epithelium (53). Somatostatin and glucagon are very poorly transported to the bloodstream following pulmonary administration due to severe local peptidase degradation (54). Protection of the amino acid terminus of peptides may inhibit peptidase attack though, leading to increased bioavailabilities (54). Pang et al. (55) showed that lung ectopeptidases were responsible for the metabolism of inhaled insulin and not insulin-degrading enzyme. Bacitracin, an ectopeptidase inhibitor, decreased the nonabsorptive loss of insulin in the isolated perfused rat lung (56). Matsukawa et al. determined the permeability coefficient of salmon calcitonin with a PEG of 5 kDa led to a thousand-fold increase in proteolytic resistance in rat lung homogenate (60). Lee et al. (61) have demonstrated the beneficial effect of PEGylation on GLP-1. The PEG conjugates were found to have 10- to 20-fold more resistance to rat lung enzymes, as compared to the unmodified version.

### Table 2  Bioavailability and $T_{\text{max}}$ of peptides and proteins following pulmonary delivery to humans using high technology inhalers

<table>
<thead>
<tr>
<th>Peptide/Protein</th>
<th>MW (Da)</th>
<th>Bioavailability$^a$ (%)</th>
<th>$T_{\text{max}}$ (hr)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LHRH analogs</td>
<td>1,200</td>
<td>18</td>
<td>1.6</td>
<td>(63)</td>
</tr>
<tr>
<td>Salmon calcitonin</td>
<td>3,400</td>
<td>11–18$^b$</td>
<td>0.3–0.7</td>
<td>(28)</td>
</tr>
<tr>
<td>GLP-1</td>
<td>3,200</td>
<td>NA$^c$</td>
<td>0.1</td>
<td>(31)</td>
</tr>
<tr>
<td>PTH(1–34)</td>
<td>4,120</td>
<td>48</td>
<td>0.2</td>
<td>(64)</td>
</tr>
<tr>
<td>Insulin</td>
<td>6,000</td>
<td>10</td>
<td>0.7–1.6</td>
<td>(65) (Exubera$^b$)</td>
</tr>
<tr>
<td>Insulin</td>
<td>6,000</td>
<td>30</td>
<td>0.2</td>
<td>(1,24) (AFREZZA™)</td>
</tr>
<tr>
<td>Human growth hormone</td>
<td>22,000</td>
<td>3.5–7.6</td>
<td>1–4</td>
<td>(29)</td>
</tr>
<tr>
<td>Erythropoietin-Fc fusion protein</td>
<td>112,000</td>
<td>NA$^c$</td>
<td>20</td>
<td>(30)</td>
</tr>
</tbody>
</table>

$^a$Relative to subcutaneous injection and dose loaded in the inhaler.

$^b$Relative to subcutaneous injection and dose deposited in the lungs.

$^c$Data not available.

Transport across the airway

Transport of macromolecules occurs across both the airway pseudostratified columnar epithelium as well as across the thin alveolar epithelium. Yet, macromolecules are absorbed into the bloodstream in larger amounts when they are delivered to the deep lung than when they are delivered to central airways (39,62). This likely originates from higher absorption rate in alveoli because of the large surface area of the alveolar epithelium as well as of the short diffusion path between the alveolar epithelium and the capillary endothelium. Fast absorption from the alveoli reduces the time of exposure to degradation processes occurring in the airspace and respiratory tissue, thereby increasing the drug fraction absorbed systemically. Bioavailabilities of proteins following pulmonary delivery range from 48% to 3.5% (Table 2), indicating that their transport across the alveolar epithelium toward the systemic circulation, in many cases, does not represent the most significant pathway in their fate.

Transport of peptides and proteins across respiratory epithelia may take place through paracellular or transcellular routes, and the main mechanism of transepithelial transport depends on the macromolecule molecular weight. Protein-specific transcytosis and peptide-specific proteolysis can enhance and reduce transport, respectively. Up to a molecular weight of approximately 40 kDa, peptides and proteins with no specific receptor on epithelial cells are transported by paracellular diffusion. Above this molecular size, nonspecific pinocytosis occurs. For instance, growth hormone (22 kDa) diffuses between alveolar epithelial cells, whereas horseradish peroxidase (40 kDa) traverses across the epithelium by nonspecific pinocytosis (66,67). Matsukawa et al. determined the permeability coefficient across the alveolar epithelium for dextrans of different molecular weights.
molecular weights (68). Dextran transport rates decreased gradually up to 40 kDa and then plateaued at 70 kDa and 150 kDa. Lowering experimental temperature from 37°C to 4°C led to 50% decrease in transport rate for dextrans up to 40 kDa, consistent with paracellular diffusion. In contrast, transport rates of 70 kDa and 150 kDa dextrans decreased by 90% when lowering experimental temperature, indicating a pinocytic transport pathway. Equivalent pore analysis based on permeability coefficients of hydrophilic solutes yielded a pore radius of 6 nm for diffusional paracellular pathways, suggesting that proteins with a radius >6 nm (~50 kDa) are excluded from paracellular transport (67).

Bur et al. assessed the transport rates of a series of serum and therapeutic proteins across primary human alveolar epithelial cell monolayers in vitro (66). Several proteins, including GLP-1, albumin, transferrin, and immunoglobulin G, were actively transported across the monolayer with higher transport in the apical to basolateral direction than in the reverse. Parathyroid hormone, insulin, and growth hormone did not show transport directionality. Although receptor-mediated transcytosis of insulin was demonstrated (69), the active process is totally saturated at therapeutic insulin concentrations and paracellular diffusion is the relevant mechanism for insulin transport (55,66). The transalveolar transport of immunoglobulin G has also been analyzed in alveolar epithelial cell monolayers and shown to involve FcRn-mediated transcytosis (70). The expression of FcRn was localized in nonhuman primate lung using immunohistochemistry and shown to be higher in epithelial cells in airways than in alveoli (71). Several therapeutic proteins were fused to the Fc-domain of an IgG1, and Fc-fusion proteins were well absorbed into the bloodstream following delivery to upper and central airways in monkeys, through Fc-Rn-mediated transport (72).

In contrast to the oral, nasal, and transdermal routes of administration, the bioavailability of a drug delivered to the lung does not systematically decrease with an increase in molecular weight (Table 2). However, similarly to other noninvasive routes of drug administration, the larger the molecular size, the slower the absorption rate and the later the time to peak plasma concentration (T_{max}; Table 2). The rate of diffusion between epithelial cells decreases with increasing molecular size (68). In contrast, the total amount of a drug absorbed from the lung depends on its biological stability during its residence within the pulmonary tissue. Large proteins cross the alveolar epithelium slowly and can remain within the alveolar space for several hours. If they undergo limited degradation within the alveoli during this time, their systemic absorption can be high. However, the comparison of, for instance, LHRH analogs and insulin indicates that the correlation between molecular weight and T_{max} is not perfect and that other parameters are involved in the pharmacokinetic profile in vivo as the elimination half-life.

**IN VITRO AND IN VIVO MODELS FOR DETERMINATION OF PULMONARY ABSORPTION**

Several models are available for the assessment of pulmonary absorption of peptides and proteins (6). These include in vitro cell cultures models, the ex vivo-isolated perfused lung model, and in vivo animal models.

In vitro cell culture models are interesting because they provide information on peptide and protein transport rates and mechanisms across respiratory epithelia and because they bring up few ethical questions. Both continuous and primary cell cultures can be used. Primary cells present cells characteristics and state of differentiation more similar to the in vivo situation than cell lines. In both cellular models, it is important that epithelial cells form a tight monolayer to represent the natural epithelial barrier. The Calu-3 cell line derives from bronchial epithelial cells of a human adenocarcinoma and is the most commonly used respiratory cell line. It can be used in both liquid-covered and air-interface conditions. Air-interface cultures are more representative of the in vivo situation where drug deposition and dissolution occur in a small volume of cell lining fluid. Calu-3 cells grown in an air-interface also shows greater similarity to airway’s epithelial morphology than liquid-covered culture (73). Most primary cell cultures consist of alveolar epithelial cells. Type II pneumocytes are isolated from normal lung tissue of humans, rats, or pigs and undergo differentiation into type I-like cells in culture. After 1 week in culture, the cells form a tight monolayer consisting mainly of type I-like cells and some interspersed type II cells (74).

In the ex vivo-isolated perfused lung model, the lung is isolated from rats, guinea pigs, or rabbits, and is suspended, together with the heart, in a humidified jacketed chamber maintained at 37°C (75). The lung is then perfused through the pulmonary artery and the perfusion solution collected from the pulmonary vein. Drugs can be delivered by the intratracheal route or by injection in the perfusate solution to simulate a systemic administration. As compared to in vitro cell culture models, the isolated perfused lung is a more complete model as structural integrity and interactions between the different cells in the lung are maintained and the impact of particle size and site of deposition within the lung can be assessed. As compared to in vivo, the isolated perfused lung allows studies on drug absorption from the lung without the influence of the other organs. However, the model does not include absorption from the airways as the tracheobronchial circulation is severed during surgery, and it demands significant surgical skills.

The most complete assessment of pulmonary absorption is provided in vivo using animal models (6). Small rodents are common models for initial studies on pulmonary drug delivery because they can be used in large numbers. Mice have been widely used for assessing pulmonary delivery of locally acting drugs. Pharmacokinetic studies following pulmonary delivery of systemically acting drugs have often been performed in rats, as blood samples at all sampling times can be collected in one rat. Guinea pigs have been widely used as an animal model of allergic asthma and infectious diseases because the airway anatomy and the response to inflammatory stimuli are comparable to the human case. Confirmatory testing can be conducted in the rabbit, the dog, the sheep, or the monkey. The dog is a good model for assessing systemic drug delivery by the pulmonary route as well as toxicity. Monkeys have very similar
anatomy and physiology as humans, but their use is restricted to advanced research. Drugs can be delivered to the animal lung by passive inhalation of an aerosol or directly in the trachea as a liquid or powder aerosol or by instillation of a liquid bolus.

SAFETY ASPECTS

There are some limitations and safety concerns to be taken into consideration when designing and delivering peptide and protein drugs to the lungs. These include local side effects, immunogenicity, and the need of a safe drug carrier.

Pulmonary administration of peptides and proteins is generally well tolerated in the short term (29, 76, 77). However, few cases allow the determination of its safety in the long term. Side effects attributed to inhaled recombinant human deoxyribonuclease I in clinical trials and post-marketing are rare (1 < 1,000) and, in most cases, side effects are mild and transient. Several Phase III trials have investigated the safety of inhaled insulin (25, 78, 79). All studies indicated that inhaled insulin was well tolerated, and the most common respiratory event reported was a mild transient cough occurring within minutes of inhalation. There was no difference in hypoglycemic events between subcutaneous and inhaled insulin. Lung function declined over the years following both injection and inhalation, consistent with aging. Yet, inhalation of insulin induced a small decrement in forced expiratory volume in 1 sec and carbon monoxide diffusing capacity but this decrement was non-progressive and reversible (79).

Following delivery of proteins, the immune defense system may recognize the native or the denatured protein as an antigen and trigger an immune response. The antibodies generated against the delivered protein can bind and neutralize it and cause the loss of protein bioactivity (80). Increased insulin antibody levels have been noted following inhalation of insulin as compared to its subcutaneous administration (79). These increased antibody levels might be related to the higher insulin doses given to the lungs (due to the reduced bioavailability) as compared to injection. Yet, these insulin-specific antibody levels were not correlated with any clinical signs. In any case, care must be given to only deliver the native protein to the lungs to reduce immunogenicity and the risk of decreased biological activity over treatment time.

Following pulmonary delivery, rapid drug absorption occurs, which may be a limitation for local treatment and may lead to multiple daily dosing. Various efforts have been made for sustaining the release of peptide and protein drugs within the lungs using carrier-based or polymer-conjugation strategies (51). Special attention should be given to the selection of the carrier or polymer for the sustained release: the carrier or polymer needs to be biocompatible, in this regard, chitosan is not adequate for pulmonary delivery as it opens tight junctions (81); large molecular mass polymers should be avoided as accumulation in the lung may occur; and high drug loading in the carrier should be achievable as masses delivered to the lungs are limited (82). Engineered or modified peptides and proteins should be considered as new chemical entities with novel biochemical properties. They may exhibit different potential risks from their unmodified version (83).

IMPACT OF SMOKING AND PULMONARY DISEASE CONDITIONS

Smoking increases insulin absorption from the lungs (84, 85). Smokers also appear less sensitive to insulin glucodynamic effects than nonsmokers following both subcutaneous injection and inhalation (84). Smoking abstinence attenuates the enhancement in pulmonary insulin absorption due to smoking, but rechallenge with a single cigarette restores it. Therefore, it is not recommended that smokers and those at risk of recidivism use inhaled insulin.

Smoking is believed to affect major alveolar clearance mechanisms in the lungs, such as absorption, alveolar macrophages, and metabolism of peptides and proteins (86–88). The mechanisms involved in the increased permeability of the lung epithelial barrier are believed to be related to changes in the integrity of tight junctions and cytoskeletal proteins (89, 90). Petecchia et al. (89) have studied the effect of exposure to cigarette smoke on tight junction’s integrity using two human bronchial epithelial cells, BEA-2B and 16HBE14o-. The exposure of the two cell lines to cigarette smoke resulted in concentration- and time-dependent tight junction’s disassembly and DNA fragmentation. Olivera et al. (90) have investigated the effects of cigarette smoke on Calu-3 airway epithelial cells. Cigarette smoke exposure led to increased polymerized actin, redistribution of the tight junction proteins from the normal apical circumferential band to a more basal localization as well as to decreased association between two tight junction proteins, thereby increasing permeability to small solutes and macromolecules. Yet, the increased permeability induced by cigarette smoke appears reversible and the lung epithelium is able to recover within a few days (86).

Chronic lung diseases have been shown to affect pulmonary absorption of proteins as well. Asthma is associated with a 30% to 40% lower absorption of inhaled insulin and with lesser glucose-lowering effects (91). However, prior administration of a bronchodilator can reverse airway obstruction and restore pulmonary insulin absorption (91). Pulmonary insulin absorption was also reduced by 35% in subjects with chronic bronchitis and by 20% in subjects with emphysema, relative to healthy subjects (92).

STABILITY ISSUES

A key issue in pulmonary delivery of peptides and proteins is the preservation of the structural and biological integrity of the therapeutic during formulation, storage, and aerosolization. Many proteins are structurally unstable and susceptible to physical and chemical degradation following exposure to various stresses as elevated temperature, extreme pH, shear strain, and surface adsorption. The dried state provides a more stable environment to the protein than the solution as shear-induced denaturation and hydrolysis and deamidation reactions are reduced (93).

Spray-drying is a fairly common process for preparing inhalation dry powders of proteins (28, 38). However, the generation of small droplets during drying provides a vast
increase in air–liquid interface, which may cause unfolding of the protein followed by aggregation. Therefore, addition of excipients to the formulation is needed to prevent physical degradation. Maa et al. have optimized the spray-drying conditions of recombinant hGH and have shown that adding the surfactant polysorbate 20 and divalent zinc ions to the liquid feed reduced the formation of hGH aggregates during spray-drying (94). Other technologies as supercritical fluid processes are also developed for the preparation of fine powders. Similarly to spray-drying, appropriate stabilizing excipients are required to retain protein stability during processing (95).

Nebulization of protein solutions in aerosols involves the formation of a large air–liquid interface and requires appropriate additives to stabilize the protein as well. Niven et al. studied the stability of lactate dehydrogenase and recombinant granulocyte colony-stimulating factor to air-jet nebulization (96). Pneumatic nebulization of lactate dehydrogenase resulted in a time-dependent loss of enzymatic activity, and the extent of inactivation was dependent upon applied air pressure and upon the volume of fluid in the nebulizer reservoir. Nebulization of recombinant granulocyte colony-stimulating factor resulted in aggregation and chemical degradation, by-products accounting for 40% of the protein after 10 min. Polyethylene glycol 1000 added at 1% w/v markedly reduced the deleterious effects of nebulization on both proteins. Thermal denaturation can be an additional degradation mechanism during ultrasonic nebulization because the protein solution warms during operation. Steckel et al. showed that aviscumine, a 57 kDa protein, lost 50% activity after 20 min of nebulization. Ultrasonic nebulization was more deleterious to the protein than air-jet nebulization (97). Yet, about 70% of the aviscumine activity could be retained by the addition of a surfactant and buffer salts. In contrast to conventional nebulizers that involve multiple recirculation of the solutions, single-pass systems that form aerosol by extrusion of the solution through a fine nozzle do not appear to cause protein denaturation (77).

CONCLUSIONS

Pulmonary delivery offers great potential for local as well as systemic delivery of peptides and proteins. Applications of local therapies are expected to expand because inhalation of drugs readily allows drug targeting to the diseased airways. Inhalation may also be an optimal route for the systemic administration of peptides and proteins particularly in the case of short-course diseases. This route of macromolecule administration has not been fully exploited yet, but the extensive development of high technology inhalers driven by inhaled insulin has paved the way toward novel applications.

The rapid pulmonary absorption of peptides and proteins may be a limitation for local therapies as it may imply multiple daily dosing. Attaining sustained drug release in the lungs is challenging because the lungs present efficient clearance mechanisms to maintain lung homeostasis and to protect the lungs from foreign substances. Although various approaches to obtain sustained release of proteins in the lungs have been tested in animal models, more work should be done to bring the most promising strategies to clinical development.

Understanding the fate of peptides and proteins in the lungs is important because fate and therapeutic action are closely linked. Future investigations should confirm the formation of protein aggregates in the lung lining fluid. The metabolism of therapeutic proteins in the lung tissue has been little studied and would deserve further investigation. Finally, studying the fate of drug carriers following delivery to the lungs could also give useful information for their optimal design.

ARTICLES OF FURTHER INTEREST

Drug Delivery: Pulmonary Delivery, p. 1164
Dry Powder Aerosols: Emerging Technologies, p. 1295
Inhalation: Dry Powder, p. 1954
Inhalation: Liquids, p. 1967
Metered Dose Inhalers, p. 2107

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