REVIEW

Department of Pharmaceutical Sciences, Wayne State University, Detroit, MI, USA

Therapeutic delivery of RNA effectors: diseases affecting the respiratory system

R. KANDIL, O. M. MERKEL

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Prof. Dr. Olivia M Merkel, Department of Pharmaceutical Sciences, Department of Oncology, Wayne State University, 259 Mack Ave, Detroit, MI 48201, USA
Olivia.merkel@wayne.edu

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Although there are several hurdles to overcome on the way to the lung, this target organ provides several advantages for successful drug absorption. Recent findings in this field of research give reason to assume that the pulmonary delivery of RNA effector molecules holds a promising potential for the treatment of numerous severe respiratory diseases.

1. Introduction

According to the World Health Organization (WHO), lung cancer (including tracheal and bronchial cancer) currently represents the fifth most frequent cause of death worldwide, infections of the lower respiratory system are on the 4th position, and COPD even on 3rd place (WHO 2014). Considering the high level of frequency and differentiation of lethal lung diseases, the great need of efficient forms of treatment becomes obvious. Besides that, other respiratory diseases like asthma or viral infections can affect patients' quality of life in an immense way as well.
After discovering the molecular pathway of RNA interference (RNAi) and the possibility of gene silencing with the help of small interfering RNAs (siRNAs), these procedures are already routinely used in genetics and genomics research and drug development today (Merkel et al. 2014). As RNAi plays an important role in the cell's defense against viral and other foreign genetic material, the biotechnological exploitation of this native defense system holds promising potential for the therapy of to date untreatable diseases. In the past years, the main research focus in the field of RNAi in the lung lay on the investigation of therapeutic approaches against lung cancer (61 %) and metastases (8 %) (Fig. 1). An especially crucial field is, however, the optimization of siRNA formulations, as described later in this article.

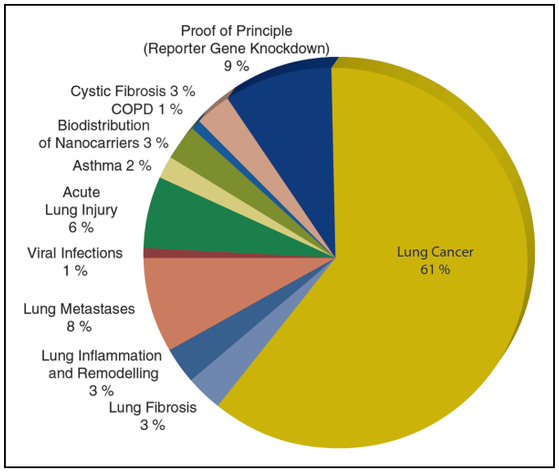

Fig. 1: Disease models described in 80 recent in vitro and in vivo studies on pulmonary RNAi published between 2011 and 2014. Reprinted with permission from Merkel et al. (2014). Copyright 2014 Elsevier.

2. Models of application

2.1. Systemic application

Unfortunately, the systemic application of siRNA drugs holds several challenges due to their unfavorable pharmacokinetic characteristics. As they are easily degraded by ubiquitous nucleases and rapidly removed from the organism *via* the kidneys, their efficient formulation is of particular importance. This is often achieved by packing the siRNA in particles or liposomes in the nanoscale. However, many siRNA delivery systems that incorporate their content through electrostatic interactions are prone to stability problems and a subsequent premature release of the embedded nucleic acids (Merkel et al. 2014). For this reason, local administration routes like the pulmonary application are becoming more and more attractive.

2.2. Local administration: target organ lung

The lung as a target organ can be reached by inhalation as well as nasal application of drugs and, therefore, provides non-invasive access which results in high patient acceptance and compliance. Beyond the known advantages of local administration routes, like reduced systemic side effects and the possibility of dose reduction, pulmonary administration features additional positive characteristics, like the avoidance of interactions with siRNA-degrading serum proteins and a comparatively low nuclease activity in the lung. Moreover, the delivered drug impacts on the lung epithelium after inhalation and is directly available in the epithelial cells after endocytosis. Those epithelial cells not only

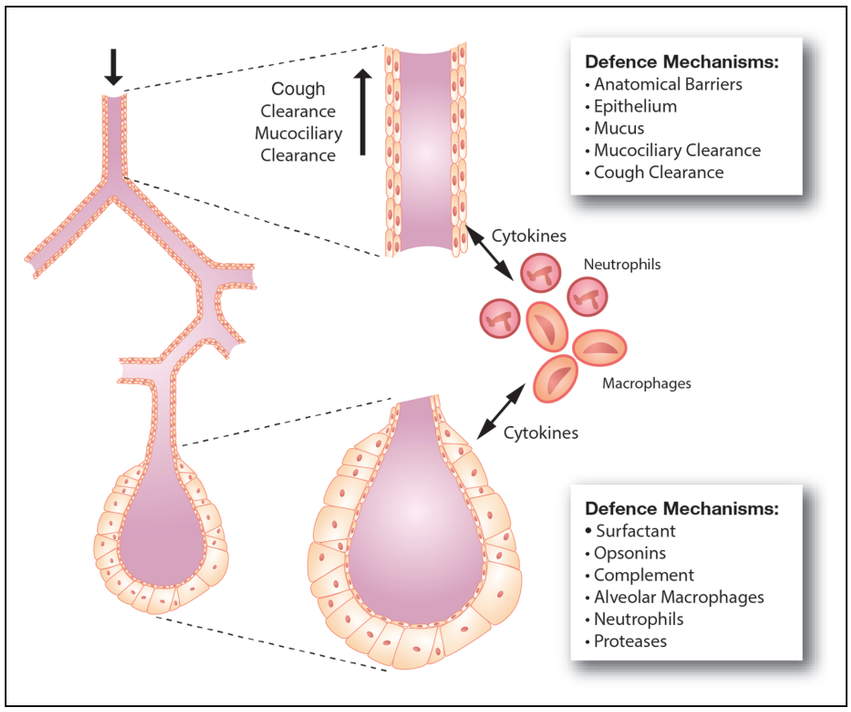

Fig. 2: Lung-intrinsic barriers to efficient pulmonary siRNA delivery. Upper magnification, trachea; lower magnification, alveoli. Reprinted with permission from Merkel and Kissel (2012). Copyright 2012 American Chemical Society.

represent an important target region concerning typical lung diseases like asthma or COPD, but are also affected by numerous viral infections, e.g. with influenza, rhinovirus, respiratory syncytial virus (RSV), or corona virus which is associated with the severe acute respiratory syndrome (SARS) (Merkel and Kissel 2012).

The large alveolar surface of the lung with its distinct vascularization and thin air-blood-barrier, furthermore, provides an ideal basis for the absorption of drugs in the systemic circulation (Merkel et al. 2014).

Despite of these advantageous aspects, the pulmonary application of RNA drugs also retains some obstacles to overcome. The air-blood-barrier only provides a low permeability for large hydrophilic and strongly negatively charged macromolecules like nucleic acids. Moreover, defense mechanisms like mucociliary clearance and recognition of exogenous particles by macrophages are problematic in this matter. Fig. 2 presents a summary of all endogenous barriers a drug has to encounter when pulmonarily applied.

3. Biological barriers in the lung

3.1. Anatomic barriers

The lung of an adult human contains about 2300 km airway, 500 million alveoles and a surface area of 75 to 140 m^2 (Gehr et al. 1978). To assure a high therapeutic efficiency, drugs have to be precisely transported to their target location and taken up by the respective cell types. The sedimentation of particles in the highly branched lung tissue depends on three different mechanisms: inertal impaction, gravitation impaction and Brownian molecular motion (Merkel et al. 2012).

While large particles with a mass median aerodynamic diameter (MMAD) > 5 μm tend to impact in the mouth and pharyngeal region as well as the upper respiratory system, smaller particles with a MMAD of 1-5 μm sediment in the lower airways and bronchioles. Very small particles with a MMAD of about 0.5 μm underlie the principles of Brownian molecular motion (Merkel et al. 2012).

The overall respected ideal droplet size for aerosols is 1 to 3 μm, as particles smaller than 1 μm are possibly exhaled without any deposition. However, recent investigations show that ultrafine particles < 100 nm are effectively accumulated in alveolar regions. This process especially takes place in asthmatic patients and is intensified by physical exercise (Chalupa et al. 2004). The size-dependent sedimentation behavior also provides an explanation for the fact that nanoparticles sediment more evenly over the whole lung, as opposed to microparticles that can easily accumulate in bifurcations of blood vessels (Kleinstreuer et al. 2008). The sedimentation of aerosol drops depends on their size, density, shape and hygroscopy, and on the breathing pattern of the patient. Because of the differences in the anatomy of the respiratory systems of humans and rodents as well as the dependence of the distribution within the lung from breath-holding maneuvers, it is challenging to examine these factors in animal models or to transfer findings from these models to humans, respectively.

3.2. Physical barriers

In the lung, secretions from the airways can trap drug particles or affect them in their stability. The airways are coated from the nose to the bronchioles with mucus, which captures exogenous materials. Ciliary cells represent about 50 % of the upper airways' epithelium and transport the mucus-moistened particles towards the oesophagus.

The velocity of this so-called mucociliary clearance is approximately 3.6 mm/min (Yeates et al. 1975) and can be even increased in diseased lungs. The eradication of foreign particles *via* expectoration supports this cleaning process. Under pathological conditions the sputum can contain DNA and actin from necrotic cells whose macromolecular character leads to an increased viscosity of the mucus. Common symptoms of acute

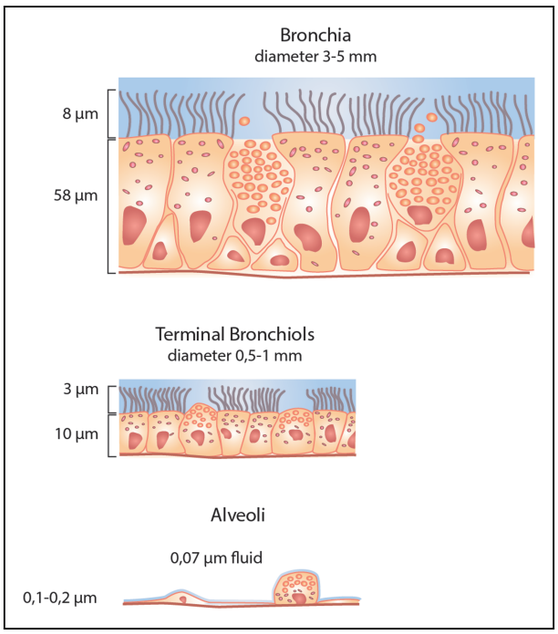

Fig. 3: As the size of epithelial cells gradually decreases from the bronchi to the alveoli, the amount of ciliated cells and mucus producing cells as well as the mucus layer thickness lessen in parallel with surfactant producing cells in the alveoli instead of mucus producing cells. Reprinted with permission from Merkel et al. (2012). Copyright 2012 American Chemical Society.

lung diseases like enhanced mucus production or an increased occurrence of oedema and cell debris can further limit the access of drugs to the target cells. The capture of the particles leads to aggregation of colloidal drug formulations like nanoparticles and impairs their mobility in a substantial way.

To improve their agility in airway secretions, certain bacteria, viruses and fungi produce enzymes that are able to cleave mucins hydrolytically and, as a consequence, reduce the mucus viscosity. Mucolytic agents like N-acetylcysteine (NAC) or rhDNase (recombinant human desoxyribonucease I) were, therefore, already experimentally examined regarding their ability to increase the mobility of nanoscale gene delivery systems in the lung.

The alveolar epithelium is not covered with mucus, but, as shown in Fig. 3, coated with a thin layer of surfactant, which is secreted by type-II-pneumocytes. Phospholipids and proteins in the surfactant decrease the surface tension at the air-blood-barrier and, subsequently, prevent the alveoles from collapsing during the expiration process. The presence of negatively charged lipids in the surfactant, however, favors the aggregation of cationic lipid-based non-viral nanoparticles and deteriorates their transfection efficiency.

Several shielding strategies can be applied to decrease this aggregation of nanoparticles. The attachment of hydrophilic non-charged polymers like polyethylene glycol (PEG) and the absorption of negatively charged polymers like poly propyl acrylic acid (PPAA) or serum albumin have already been tested, among others. These approaches were able to reduce the positive surface charge of the delivery systems and, therefore, their interactions with bronchoalveolar lavage fluid (BALF) and alveofact (Kleemann et al. 2005).

Besides that, "tight junctions" between the epithelial cells of the respiratory system limit the access of nucleic acid containing particles from the basolateral side. However, it was observed that those can be reversibly opened by polymer drug carriers like poly acrylic acid (PAA).

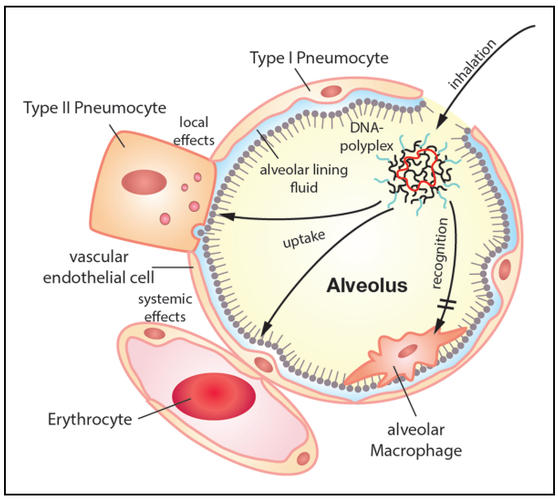

Fig. 4: After entering the alveoli, gene delivery systems can possibly interact with the alveolar linage fluid or can be taken up by various cell types. Recognition by and uptake into macrophages should be avoided, for example, by adjusting the size and surface of nanoparticles. Uptake into pneumocytes could lead to local therapeutic effects, and transcytosis into the systemic circulation could lead to systemic wanted or unwanted effects. Reprinted with permission from Merkel et al. (2012). Copyright 2012 American Chemical Society.

3.3. *Immunological barriers*

While the mucociliary clearance remains the most effective cleaning mechanism in the upper airways, exogenous particles are mostly removed from the alveoles *via* phagocytosis by macrophages and epithelial endocytosis (Fig. 4).

According to their size of 15 to 22 µm, alveolar macrophages phagocytose particles with a diameter of 1 to 3 µm efficiently (Chono et al. 2006), while they appear to ignore nanoparticles < 260 µm (Lauweryns et al. 1977). The latter can be transported to the systemic circulation by caveolae-mediated endocytosis. The uptake of nanoparticles can induce an enhanced secretion of proinflammatory cytokines, leading to an influx of polymorphic neutrophil granulocytes (PNMs), the generation of reactive oxygen species (ROS) and DNA double strand breaks (Merkel et al. 2012).

Other immune responses that provide the invasion of exogenous material in the lung are soluble components of the immune system, like opsonins, complement factors and antibodies. Ultrafine particles, as compared to bigger colloids, more severely result in cytotoxicity as well as allergic and inflammatory responses (Merkel et al. 2012).

3.4. *Metabolic barriers*

Nanoparticles soluble in extracellular fluids or lipid membranes are rapidly absorbed through the epithelial membrane of the lung, while smaller hydrophilic drugs are taken up by active transport systems. Larger hydrophilic substances like DNA or insoluble materials are, however, moved to the systemic circulation or other organs (Merkel et al. 2012). To achieve a systemic effect, this transport is favorable, but regarding local therapy it leads to a reduction of the active dosage and, consequently, of the potential therapeutic effect. It was recently shown that zwitterionic nanoparticles (net charge equals zero) with a diameter < 34 nm are quickly evacuated to the lymph nodes, while larger particles remain in the lung. Conversely, about half of such zwitterionic particles with a diameter < 6 nm were taken up into the systemic circulation before their excretion by the kidneys (Choi et al. 2010). Hence, the particles' behavior after pulmonary application is strongly dependent on their size. A

further parameter playing an important role is the particles' surface charge, as it influences interactions of the colloidal systems with extracellular fluids which can, e.g., affect the stability of the particles.
Aside from the acid proteinase I, the alkaline proteinase II and a very few other peptidases, the lung overall represents an organ of very low metabolic activity, which provides a crucial advantage for the pulmonary administration of nucleic acids.

4. Requirements of delivery systems for RNA-based drugs

On the basis of the depicted barriers and hurdles, the optimal vehicle for pulmonary delivery is expected to be neither cytotoxic nor immunogenic and ideally biodegradable. It has to protect the incorporated RNA-based drug from degradation by nucleases during its transport and should be able to maintain high drug concentrations over a long time period by preventing both the recognition by macrophages and the rapid excretion *via* the kidneys. Furthermore, the RNA should only be bound in a reversible way to ensure an efficient release at the target region. The ideal carrier would, moreover, specifically target certain cell types and mediate the uptake of the RNA-based drug. Once arrived in the target cell, the RNA ultimately has to be released from the endosome and, in case of siRNAs, be gathered in the RNA-induced silencing complex (RISC) (Merkel et al. 2012) (Fig. 5).

5. Formulations for pulmonary delivery

5.1. Modification of the RNA

For the mediation of RNAi, different types of nucleic acids can be employed. In most cases, chemically synthesized siRNAs or shRNAs are used, which are expressed by plasmid DNAs. Because of the high susceptibility for degradation by nucleases, chemical modifications of the sugar, the phosphate backbone or the bases of RNA are implemented in order to increase the in vivo stability. A key modification is the 2'-*O*-methylation of the ribose, which can minimize off-target-effects and immune stimulating reactions without reducing the efficacy of the siRNA (Sioud et al. 2007) (Fig. 6).
A further possibility to modify RNA is presented by the length of the double strands. While siRNAs are usually applied as 19 base pairs long duplexes with 2 nucleotide overhangs at the 3'-end and, therefore, resemble the naturally occurring processing products of the cellular RNase Dicer, 2'-*O*-methylated so-called Dicer-substrate siRNAs (DsiRNAs; 25-27 base pair long duplexes) can be up to 100 times more potent (Kim et al. 2005).
Moreover, cholesterol-conjugated siRNA with a phosphorothioate backbone as well as RNAs with a modified sugar (e.g. locked nucleic acids, LNA) have already been applied successfully (Merkel et al. 2014).

5.2. Chemistry of the carriers: polymers

Although viral vectors own certain advantages for the cellular transport of nucleic acids, there are still some safety restrictions concerning cell toxicity, immunogenicity, carcinogenicity and uncontrollable virus replication. Besides that, their reproducible formulation remains a challenging task. Non-viral vectors present the advantage that they are chemically modifiable and, therefore, can be tailored to the exact requirements for RNA delivery. Another option is the application of "naked" (chemically modified) RNA. Although already applied successfully, most authors of current publications decided to package nucleic acids in form of nanoparticles (Merkel et al. 2014). However, several cellular and intracellular barriers that viruses naturally overcome have to be considered during the formulation with carrier materials. Here, the biggest hurdles are presented by the cell membranes as well as intracellular membranes which an artificial carrier has to transit. Most nanoparticles are taken up into the cells by endocytosis, however, they are then located in the endosome which is surrounded by a membrane. Since siRNAs can only develop their effect in the cytosol, the endocytosed nanoparticles have to be released from the endosome to the cytosol. In the past years, the cationic polymer polyethylenimine (PEI) was frequently utilized with a preferable molecular weight between 5 and 25 kDa. Polymers with higher weights can increase the cytotoxicity due to their enhanced aggregation on cell surfaces (Fischer et al. 1999).
PEI with a lower molecular weight may be less toxic, but it is also less effective as a transportation vector. On the one hand, due to their smaller amount of positive charges, it is more difficult for smaller PEIs to tightly incorporate negatively charged nucleic acids. On the other hand, with a too low overall surface charge, it becomes almost impossible to induce cellular uptake by charge-mediated interactions with the cell membranes (Merkel et al. 2012).
The high positive charge density of PEI efficiently condenses the negatively charged nucleic acids into consolidated complexes and, therefore, protects them from degradation by nucleases. Furthermore, PEI holds protonable amino groups that can be used by the polymer to change its protonation state upon pH changes in the cytosol. Notably, PEI binds protons inside of endosomes due to its high buffering capacity, which is referred to as "proton-sponge"-effect. This characteristic of PEI leads to an osmotic swelling and, consequently, to the rupture of the endosomal membrane which results in the release of the PEI/nucleic acid-complex to the cytosol (Boussif et al. 1995).
PEI polymers can be categorized into those with a branched and those with a linear architecture. Strongly branched PEI shows an enhanced complex formation with DNA and RNA and forms smaller complexes than linear types do.
To reduce the PEI's toxicity, several approaches were tested, including polyethylene glycol (PEG) polymerized and biodegradable PEIs. The incorporated PEG might be able to reduce the cell toxicity drastically, however, at the same time it can lead to a decrease in transportation efficiency *in vivo*, presumptively due to the reduced interactions with cell surfaces and the associated deterioration of the cellular uptake (Merkel et al. 2012).
Another approach is to package the nucleic acids in polysaccharide-based materials. Chitosan, a family of linear polysaccharides consisting of D-glucosamine and N-acetyl-D-glucosamine, is a biodegradable example. It was successfully utilized *in vivo* for the transport of genetic material to mucosal tissue (Koping-Hoggart et al. 2001). Compared to PEI, chitosan provides a lower buffering capacity and can, therefore, not function as a sponge for protons in the endosome. Nevertheless, due to its high biocompatibility and mucoadhesive characteristics, chitosan still is a potential transportation vector for nucleic acids. By combination with PEI, the solubility and biocompatibility as well as the cellular uptake of Chitosan particles can be improved (Mao et al. 2005).
One of the earliest polymers that have been examined as a delivery system for nucleic acids is poly-L-lysine (PLL). Based on its high toxicity, only little transfection activity can be achieved with PLL formulations, especially when using PLLs with a molecular weight higher than 25 kDa (Merkel et al. 2012). To reduce this toxicity, PEG or polylactic-co-glycolic acid (PLGA) can, among others, be attached to the PLL backbone.

Due to its biodegradability and biocompatibility, PLGA has already been approved for certain clinical applications, like absorbable suture material or bone implants, by the U.S. Food and Drug Administration (FDA). It is extensively applied for the transport of drugs and genetic material, though not specifically developed for the latter field of application. It therefore possesses several disadvantages like low encapsulation efficiency, nucleic acid degradation during the hydrolysis of PLGA and slow release kinetics. Modifications of the PLGA particles to overcome these hurdles are e.g. precondensation with the help of cationic polymers like the already mentioned PEI or chitosan to improve the incorporation of the nucleic acids (De Rosa et al. 2003).
Recently, the main focus of research lies not only on the successful delivery of the nucleic acid material into the target cells, but also on an enduring and controlled gene expression (for gene vectors) or down-regulation (for siRNAs). For this matter, polymeric nanoparticles based on PLGA or other biodegradable polymers provide numerous possibilities for the future pulmonary nucleic acid transport.

5.3. Surface modification and active targeting

PEIs can be modified with the cell-penetrating peptide TAT (Kleemann et al. 2005), biotinylated TAT-RGD (Renigunta et al. 2006) or galactose (Chen et al. 2008) in order to improve their interactions with the cell surface and, ultimately, their cellular uptake. For active targeting approaches polyplexes are linked with a ligand that is supposed to interact with a specific target structure on the cell surface. This target structure should preferably be present on the desired target cell type in a selective and overexpressed way to achieve a specific and efficient uptake of the particles.
Apart from liposomes and viral vectors, also polymeric non-viral vectors are utilized for active targeting attempts, with PEI and PLL leading the way. One way of functionalizing these materials is to couple them with sugars or sugar derivates. Glycosylated polymers have already been examined as potential therapeutic formulations for cystic fibrosis (Kim et al. 2004) and lung cancer (Grosse et al. 2008). Unfortunately, no distinct lung specificity of the conjugates could be detected so far, when not applying them directly into the lung.
In most active targeting approaches, the target structure consists of an internalizing receptor on the surface of the target cells that normally binds its natural ligand or antibody. For lung cells, for example, folate is an attractive binding partner, as these cells overexpress the respective receptor. Likewise, lactoferrin as a receptor ligand for bronchial epithelial cells (Elfinger et al. 2007) and anisamid as a ligand for the epidermal growth factor receptor (EGFR) (Li et al. 2008) have been tested.
Furthermore, regarding the specific targeting of lung tissue, antibodies against the platelet endothelial cell adhesion molecule (PECAM-1 or CD31) (Li et al. 2000) and fab fragments of polyclonal antibodies against the polymeric immunoglobulin receptor (Ferkol et al. 1995) have been investigated.
The results of these studies give reason to assume that targeted delivery of nucleic acids in the lung is possible and holds potential for the treatment of respiratory diseases. The greatest probability of success is promised by nanoparticles with a ligand directed against an internalizing receptor overexpressed by the target tissue in combination with a local application in form of aerosols.

6. Administration routes

Among the different approaches that can be used for the successful nucleic acid delivery to the lung, inhalation is the most clinically relevant one. The difficulty in developing respective formulations especially lies in maintaining the integrity of vehicle and drug. In case of dry powders, procedures like lyophilization or spray drying are usually needed. Those can, however, lead to aggregation of the particles and, consequently, to biological or physicochemical instability of the drug. Impairments of the biological activity can especially emerge from strong shearing forces the formulation is exposed to during the process of aerosolization (Nielsen et al. 2010). Moreover, the precedent nebulization of solute substances and the adhesion of positively charged nanoparticles to plastic parts of the inhalers can limit the drug dose severely (Merkel et al. 2014).
Another local and little invasive possibility is the application of nasal drug suspensions. The disadvantage of this method is the less quantitative delivery to the lung, as part of the administered dose inevitably gets lost by deposition in the nasal cavity or by swallowing.

7. Clinical implementation

With decoding the human genome, sequences of various genes became accessible that had previously been regarded as "undruggable". The discovery of the RNAi was associated with the hope to be able to address those disease-related genes by therapeutic knockdown. SiRNA-based therapeutics have the potential to specifically inhibit any gene as long as its sequence is known. In practice, however, it is still a challenging task to transfer this promising approach into clinical applications. As cells lack a capable uptake mechanism for nucleic acids, the efficient import over biological barriers in general, as well as the directed delivery to specific target organs and cells, remains one of the biggest challenges for the therapeutic utilization of RNA drugs *in vivo*. Despite all hurdles, there are several promising siRNA-based therapeutics being currently evaluated in clinical studies. While polymer and lipid formulations are primarily applied i.v., local application routes are preferred for "naked" siRNA drugs in most cases (Khatri and Misra 2012).

8. Conclusion

Altogether, current developments give reason to assume that inhalable formulations for pulmonary RNA delivery are going to be realized in the future. The biggest hurdles on the way to clinical application are the absence of biocompatible carriers that are able to overcome extra- as well as intracellular barriers, and the yet inefficient selective delivery of RNA-based drugs to the target cells.

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