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Non-viral siRNA delivery to the lung ☆

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Abstract

SiRNAs exert their biological effect by guiding the degradation of their cognate mRNA sequence, thereby shutting down the corresponding protein production (gene silencing by RNA interference or RNAi). Due to this property, siRNAs are emerging as promising therapeutic agents for the treatment of inherited and acquired diseases, as well as research tools for the elucidation of gene function in both health and disease. Because of their lethality and prevalence, lung diseases have attracted particular attention as targets of siRNA-mediated cures. In addition, lung is accessible to therapeutic agents via multiple routes, e.g., through the nose and the mouth, thus obviating the need for targeting and making it an appealing target for RNAi-based therapeutic strategies. The clinical success of siRNA-mediated interventions critically depends upon the safety and efficacy of the delivery methods and agents. Delivery of siRNAs relevant to lung diseases has been attempted through multiple routes and using various carriers in animal models. This review focuses on the recent progress in non-viral delivery of siRNAs for the treatment of lung diseases, particularly infectious diseases. The rapid progress will put siRNA-based therapeutics on fast track to the clinic.

Keywords: RNA interference; siRNA delivery; Lung

Contents

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1. Introduction

RNA interference (RNAi) is a post-transcriptional gene silencing phenomenon wherein double-stranded RNA (ds-RNA) triggers the degradation of a homologous messenger RNA (mRNA), and is believed to play critical roles in defense against viral attack [1]. More specifically, in an initiation step, exogenous (e.g., viral) ds-RNA is digested by the enzyme Dicer, a member of the RNase III family of ds-RNA-specific ribonucleases [2,3], into short interfering RNAs (siRNAs) of 21–23 bp in length. In the effector step, the antisense strand of siRNAs is incorporated into RNA-induced silencing complex (RISC). The RISC then targets the mRNA complementary to the siRNA within the complex, cleaves the target mRNA, and thus lead to inhibition of mRNA translation. In mammalian cells, RNAi can be triggered by synthetic RNA duplexes that are 21–23 nt long [4,5]. They guide the degradation of the cognate mRNA sequence, hence halting the corresponding protein production [4,5]. SiRNA may be obtained by chemical synthesis or expressed from a DNA-vector [6,7]. In the latter, a DNA insert of about 70 bp encoding a short hairpin RNA (shRNA) targeting the gene of interest is cloned into an expression vector. When the insert-containing vector is transfected into the cell, it expresses the shRNA which is rapidly processed by the cellular machinery into a 19–22 nt double-stranded RNA (ds-RNA). Because RNAi confers transient interference of gene expression in a sequence-specific manner, it represents a new class of nucleic acid-based molecules likely to have significant medical utility. In fact, RNAi is viewed as one of the most important recent discovery in biology [8].

Due to the generality of this phenomenon, siRNA sequences could be designed for virtually any target mRNA. This methodology could be exploited for therapeutic use or as a research tool in many ways. For example, the protein of interest could be a mutated human protein, as in the case of Huntington's disease (HD), which is an autosomal dominant inheritable neurodegenerative disorder currently without effective treatment, caused by an expanded polyglutamine (poly Q) tract in the corresponding protein huntingtin; suppressing the huntingtin expression in neurons in the brain is expected to delay the onset and mitigate the severity of HD [9,10]. In other instances of RNai, the protein of interest could be foreign, as in the case of influenza infection [11] or SARS [12], where the target proteins are encoded by the viral genome. In addition, siRNAs could be used as a research tool for the elucidation of gene function [13]. Compared to expensive and time-consuming conventional gene knockdown experiments, siRNA-based gene silencing is cheaper, faster, and can be used to establish the biological function and phenotypic effects of many genes involved in both health and disease [1,14–16].

Because of their size and chemical degradability under physiologically relevant conditions, delivery of siRNAs usually requires a vector or carrier for their transfection into mammalian cells. Many non-viral vectors previously tested for DNA delivery have been adapted for siRNA since the barriers to the delivery are similar.

Due to its location and physiological function, lung is susceptible to many diseases. For example, it is exposed to many environmental pollutants including pollen, smoke, ozone, and volatile organic compounds that lead to diseases such as asthma and cancer. Furthermore, many of the lethal infectious diseases such as influenza and SARS are airborne and use lung as the target site or the main entrance to the body. Given their lethality and prevalence, lung diseases have attracted particular attention as targets of siRNA-mediated cures. In addition, since lung is accessible to therapeutic agents via multiple routes, such as nose and mouth, thus obviating the need for targeting, it has been a convenient model for the *in vivo* validation of siRNA-mediated therapeutic gene silencing.

2. Intracellular and extracellular barriers to siRNA delivery

The negative charge and chemical degradability of siRNA under physiologically relevant conditions make its delivery a major challenge. Owing to the chemical similarity between DNA and RNA, the barriers to their delivery are likely similar. Those to DNA have been extensively investigated in the context of gene therapy and may be grouped into intracellular and extracellular barriers [17,18]. Both types of nucleic acids must cross the cell membrane and successfully escape lysosomal degradation. The negative charge and size of siRNA and plasmid DNA (typical molecular weights of some 15 kDa and 1600 kDa, respectively) imply that they cannot bind to the cell surface or cross the cell membrane by passive diffusion. However, while plasmid DNA needs to enter the nucleus to initiate transcription of the encoded genes, siRNA can function in the cytosol. Therefore, achieving therapeutic effect by the delivery of nucleic acids to non-dividing cells may be more easily achieved with siRNA than with DNA.

The extracellular barriers to siRNA delivery depend upon the route of administration (e.g. intravenous (i.v.), intranasal (i.n.), intratracheal (i.t.), subcutaneous, intratumor, intramuscular, or oral) which, in turn, depends upon the targeted disease. In the case of i.v. delivery for example, the first major extracellular obstacle is its degradation by serum nucleases, which may be overcome by complexation with a suitable agent. Such complexation agents should also facilitate cellular uptake and endosomal escape [19]. Since charge complementarities aid complex formation, to bind negatively charged siRNA such carriers are typically positively charged. Non-viral siRNA transfection carriers can be categorized into lipid- and polycation-based ones. Lipofectamine 2000 [20,21] and cardiolipin analogues [22,23] have been successfully used for the delivery of siRNA. In some cases, lipid-mediated siRNA transfection is shown to be at least a thousand fold more efficient than that of naked siRNA [21]. During siRNA/DNA transfection mediated by cationic lipids, negatively charged nucleic acids and positively charged lipids spontaneously form nanoparticles (known as lipoplexes) of 50–200 nm in diameter [24–26]. In the absence of serum, these particles have positive surface charges, hence favoring their interaction with cells and making them suitable for efficient siRNA delivery, although interaction with serum components may be

a major factor that influence their performance when used systemically [26].

Bioavailability, cell-type-specific delivery, and *in vivo* pharmacokinetics (depending on the routes of administration) are important parameters that decide the *in vivo* gene silencing efficacy. Unfortunately, information about the biodistribution of naked or formulated siRNA and its chemically modified analogues is rather scarce [27–29]. Lipid-mediated delivery of an siRNA against apolipoprotein B (ApoB) has been used to target ApoB mRNA in the liver. Whereas administration of the *Apob*-specific siRNA siApoB-1, without formulation or chemical conjugation, at doses higher than 50 mg/kg was previously shown to have no *in vivo* silencing activity [30], ~80% silencing of liver *Apob* mRNA and ApoB-100 protein was achieved with a single 1 mg/kg dose of lipid-formulated siApoB-1 in a non-human primate [28]. The lipid formulation in this case contained the lipids 3-*N*-[(ω-methoxypoly(ethylene glycol)$_{2000}$)carbamoyl]-1,2-dimyristoyloxy-propylamine (PEG-C-DMA), 1,2-dilinoleyloxy-*N*,*N*-dimethyl-3-aminopropane (DLinDMA), 1,2-distearoyl-*sn*-glycerol-3-phosphocholine (DSPC) and cholesterol, in a 2:40:10:48 molar ratio. A major fraction of the siRNA delivered intravenously using this vector accumulated in the liver, followed by spleen and small intestine with some 40-fold lower accumulation.

Similar to lipid-based transfection carriers, the positive charges of polycations allow an efficient interaction with siRNAs to form polyplexes which can bind onto cell plasma membrane and be endocytosed. Unlike lipoplexes that rely on the fusogenic property of the liposomes to mediate endosomal escape, polymeric carriers such as polyethylenimine (PEI) [31–34] use the so-called "proton sponge" effect to enhance endosomal release of endocytosed polyplexes [19,35–37]. According to this mechanism, the unprotonated amines with different pK_a values confer a buffering effect over a wide range of pH. This buffering may protect the siRNA from degradation in the endosomal compartment during the maturation of the early endosomes to late endosomes and their subsequent fusion with the lysosomes. The buffering property also allows the polycation polyethylenimine (PEI) to escape from the endosome: at lower pH, the buffering by PEI causes an influx of chloride ions and water into the endosomes, which eventually burst due to increased osmotic pressure, thus facilitating intracellular release of PEI–siRNA polyplexes. High cation density of PEI (a potential positive charge per 43 Da, which is the monomer's molecular weight) also contributes to the formation of highly condensed particles by interacting with nucleic acids. However, it also results in significant cytotoxicity, particularly for large PEIs [34,35,38,39].

3. Barriers to siRNA delivery to the lung

As with any other nucleic acid, in the specific case of siRNA's delivery to the lung the extracellular barriers depend upon the route of administration. Introduction of the complexes to the lung could be achieved, in principle, by i.n., i.t. or systemic routes. The first two are particularly appealing since they offer a unique opportunity for the specific delivery of siRNAs to airway and alveolar epithelium. A large body of literature and experience in gene delivery to the lung supports the feasibility of this approach [40]. However, airway-directed gene delivery is not simple because the lung has evolved both physical and immunologic barriers that can hinder effective transduction of epithelial cells [40]. Besides such physical phenomena as cilia beating and mucociliary clearance, the possible interaction of the complexes with the airway surface liquid (ASL) covering the airway epithelial cells poses another major barrier [41]. The major constituents of ASL, phospholipids, proteins, and mucins (high-molecular-weight glycosylated proteins), could affect nucleic acid transfer efficiency. First, negatively charged ASL constituents could directly bind to positively charged complexes, altering their size and switching their overall charge to negative, and hence affecting their diffusion to the target cells or cellular uptake. Additionally, binding of negatively charged ASL components might displace the nucleic acid from the complexes and consequently lower their delivery efficiency. In fact, lung surfactants have been shown to impede cationic liposome-mediated DNA transfection. For example, Alveofact, a natural surfactant extracted from bovine lung lavage, inhibited gene transfer mediated by cationic liposomes *in vitro* [42]. Interestingly, polycation-mediated gene transfer was affected neither *in vitro* nor *in vivo* by the surfactant [43]. In contrast, bronchoalveolar lavage fluid (BALF), collected by bronchoscopy, inhibited both liposome- and polycation-mediated gene transfer [41]. The major inhibitory effect in this case was shown to be caused by adsorption of BALF proteins to the complexes and the resultant alteration of their surface charge, and not due to the release of DNA from the complexes. In addition, in patients with diseases such as cystic fibrosis characterized by a lung pathology with thick mucus production, direct delivery into the lung via intranasal or intratracheal routes is likely to be more challenging [29,44].

A recent study has shown that cationic lipid-mediated siRNA transfer to airway epithelial cells *in vivo* is inefficient [29]. Following *in vivo* lung transfection using a Genzyme (http://www.genzyme.com) lipid (GL67) in mice, siRNAs were only visible in alveolar macrophages. SiRNAs targeted to β-galactosidase reduced β-gal mRNA levels in the airway epithelium of K18-lacZ mice by 30%. However, this was insufficient to reduce protein expression.

Considering the foregoing difficulties, systemic administration may be perceived as an alternative to intranasal and intratracheal administration of the complexes. Information on the biodistribution of the complexes of siRNA with cationic liposomes or polycations after systemic administration is rather scarce [28]. Owing to the similarities between siRNA and DNA, considering the case of the complexes of DNA would be instructive. Biodistribution of the latter is a complex process dependent on their colloidal properties, as well as interaction with blood components. For example, in experiments involving gene delivery to the liver, DNA–polycation complexes of 100–200 nm in diameter, unlike larger complexes, are transported into parenchymal cells. Furthermore, scavenger cells usually absorb complexes bearing strong anionic charge, resulting in

elimination of the genetic material from the body. A strong positive charge on the complexes can also be deleterious. For example, following intravenous administration in mice, 60% of the dose of plasmid DNA delivered as a complex with cationic liposomes accumulated in the liver; at the same time, the level of transgene expression per microgram of DNA taken up in tissue was a 1000-fold lower in the liver than in the lung. Apparently, the decreased transgene expression in the liver is due to the rapid degradation of DNA following phagocytosis of the lipoplexes by the Kupffer cells [45].

The distribution of transgene expression following administration of DNA–polycation complexes is similar to that described above. Expression is primarily observed in the lungs and to a much lesser extent in such other major organs as spleen, liver and heart [44,46]. Interestingly, transgene expression is significantly altered when plasmid DNA is formulated with polycations grafted with hydrophilic polymers, such a Pluronic. In this case, the expression of the reporter gene was evenly distributed among the aforementioned major organs [45]. Similar to the case of DNA, i.v. injection of siRNA with PEG-containing liposomes has also been reported to result in preferentially delivery of siRNA to the liver [28] implying that the surface properties of the polyplexes is an important factor that dictates their *in vivo* distribution.

Preferential gene expression in lungs upon i.v. delivery when DNA is complexed with unmodified cationic liposomes or polycations is rather fortuitous in the context of lung-affecting diseases and may be exploited for siRNA delivery in the treatment of lung diseases. It is noteworthy that intranasal delivery of naked siRNA has been shown to result in therapeutic gene silencing, and is as efficient as that with non-viral vectors. A plausible reason for the success of this method is that degradation of siRNA by nucleases is not significant in the airway [29] unlike other routes such as i.v. wherein no significant gene silencing has been observed with naked siRNA [32,34,47].

4. Infectious diseases of the lung targeted with non-viral siRNA delivery

4.1. Influenza virus infection

Influenza virus causes one of the most prevalent infections in humans. In a typical year, 10–20% of the United States population is infected by this virus, resulting in up to 40,000 deaths and 200,000 hospitalizations (http://www.cdc.gov/flu/). An influenza pandemic is caused when a new strain of the virus – one to which humans have no immunity – acquires the ability to readily infect people. Based on a global population estimate of 6.5 billion people and the estimated mortality rate of the Spanish flu pandemic of 1918 [48], the next pandemic might kill at least 75 million people. If the pandemic strain proves to be the highly virulent H5N1, the numbers could be much higher [49]. Current vaccines can prevent illness in 70–80% of healthy individuals under the age of 65, but the protection rate drops for those most susceptible to infection, namely elderly [50], infants [50,51], and individuals with a weakened immune system. Furthermore, influenza vaccines need to be formulated each year because the viral antigens (HA and NA) that elicit the neutralizing antibodies change, rendering the previous year's vaccine ineffective against new subtypes. Since it takes about 6 months to produce the vaccine, a decision must be made in advance as to which virus strains to include, which constrains the applicability of vaccination in the face of an approaching epidemic. In addition, shortage of vaccines in a flu season often arise due to technical difficulties in the vaccine production process. For example, the Chiron Corporation could provide none of its influenza vaccine (Fluvirin®) for distribution in the United States for the 2004–2005 flu season [52]. The Medicines and Healthcare Products Regulatory Agency (MHRA) in the United Kingdom, where Fluvirin® is produced, suspended Chiron's license to manufacture this vaccine in its Liverpool facility for 3 months, thus preventing any release of this vaccine for the influenza season. This action, prompted by the vaccine's contamination with a deadly bacterium, cut roughly by half the expected supply of trivalent inactivated vaccine (flu shot) available in the United States that winter. This mishap underscored the need for alternative strategies for the prevention and treatment of influenza.

Although four antiviral drugs have been approved in the United States for treatment and/or prophylaxis of influenza [53], their use is limited by concerns about side effects and the risk of emergence of drug-resistant strains [52].

Influenza virus is an enveloped virus of the Orthomyxovirus family. Influenza virions are usually roughly spherical and about 200 nm in diameter. The viral genome is composed of eight segments of single-stranded RNA of negative polarity [54,55] coated with RNA-binding proteins (the nucleocapsid protein, NP) to form the nucleocapsid. Overlying the nucleocapsid is a layer of the matrix protein M1 which, in turn, is surrounded by viral envelope derived from the host cell membrane. The virus is classified as influenza A, B, and C types based on the differences in NP and M1. Viruses belonging to type A are further classified into subtypes according to the serotype of the HA and NA proteins [56]. Influenza type B and C infections cause a mild respiratory illness and are not thought to cause epidemics.

4.1.1. Polycation-mediated intratracheal or intranasal delivery of siRNA inhibits influenza in mice

We found that siRNAs specific for conserved regions of influenza virus genome are potent inhibitors of influenza infection both *in vitro* [11] and *in vivo* [47]. To design siRNAs that are effective against influenza, we focused on those regions of the viral genome that are conserved among different subtypes and strains of virus from humans, swine, equine, ducks, and chickens, despite antigenic drifts and shifts. A total of 20 siRNAs specific for NP, PA, PB1, PB2, M2, and NS genes were designed [11]. HA and NA were not selected for siRNA targeting due to extensive variation in these genes among different virus isolates.

The ability of synthetic siRNAs to inhibit influenza virus production in cell culture was tested by introducing siRNA into the cells before and after virus infection by electroporation and assaying virus titers in the culture supernatants at different times after the infection; it was also verified in chicken embryos. In

the latter case, siRNA was introduced as a complex with the commercial liposomal reagent oligofectamine [11]. The following conclusions were drawn from these studies: (i) influenza virus production can be inhibited by siRNAs specific for different viral genes, especially those encoding NP, PA, and PB1; (ii) inhibitory effect of siRNA is observed in cells that were infected with the virus prior to the introduction of siRNA; (iii) siRNAs that inhibited influenza viral production in cultured cells also inhibited viral production in chicken embryos; and (iv) while M-siRNA specifically inhibited the accumulation of M-mRNA, siRNA specific for NP and PA abolished the accumulation not only of the corresponding mRNA, but also of vRNA and cRNA, suggesting a critical role of NP and PA proteins in viral transcription and replication. Both the targeted mRNA degradation and the resulting global inhibition of viral RNA transcription make the NP- and PA-specific siRNAs especially potent inhibitors of influenza viral infection.

We then set out to test the feasibility of influenza inhibition in mice by PEI-mediated siRNA delivery. As a model, we first tested the ability of jetPEI (a proprietary linear PEI formulation from Polyplus-Transfection, Illkrich, France) to deliver the Luc (luciferase) gene into the lungs of mice and also the efficacy of jetPEI/Luc siRNA complex to inhibit the expression of Luc in the lungs [47]. Consistent with previous findings [44,46], when jetPEI/DNA complexes were injected i.v. through the retro-orbital vein Luc activity was highest in the lungs where it could be detected for at least 4 days; in heart, liver, spleen and kidney, Luc activity was 100–1000 times lower (depending upon the PEI/DNA ratio) and detectable for shorter periods after injection. When jetPEI/DNA complexes were administered i.t., the luciferase expression in the lungs was about 25-fold lower. Luc-siRNA/jet PEI complexes delivered i.v. resulted in a 17-fold suppression of the expression of the Luc gene delivered i.t. in complex with jetPEI. GFP-siRNA/jetPEI (GFP = green fluorescent protein) complexes had no effect on the expression of Luc. Since Luc-siRNA can inhibit the Luc expression only in those lung cells that were transfected with the Luc-DNA, these results indicate that jetPEI delivers siRNA effectively into the lung cells of mice, and hence delivery of appropriate siRNA/jetPEI complexes may be used to inhibit influenza infection in the lung. The reason for the relatively higher delivery of the complexes of DNA and siRNA with PEI is not clear but may be because the lung (i) contains the first capillary bed traversed by i.v.-injected materials or (ii) is among the most vascularized tissues in the body. We also noticed that the relative expression in the lung compared to other organs was higher when the surface charge of the complexes is cationic (positive zeta potential).

Significant (~10-fold) reductions in virus titers were observed when 60 μg of NP- or PA-siRNA in complexes with jetPEI were administered i.v. 3 h before infecting mice i.n. with 12,000 pfu (plaque forming units) of influenza virus (Influenza A/Puerto Rico/8/34 or PR8, subtype H1N1). No reduction in virus titer was observed if these siRNAs were administered in PBS or when GFP-siRNA was used in complex with jetPEI. When complexes of NP- and PA-siRNAs (60 μg each) with jetPEI were injected together, even greater, some 100-fold, reduction in virus titer was observed. Although a 10-fold effect may seem modest, lung virus titer reductions of this magnitude usually accompany survival of lethal challenge in vaccine development studies. This is particularly significant considering that a challenge dose of the virus used (12,000 pfu) was far greater than those likely encountered upon natural exposure of humans to influenza virus. Significant reduction of virus titer was observed even when the siRNA complex was administered 12 h before infection, or when the lungs were analyzed 2 days after infection [47]. These results imply that siRNA in complex with an appropriate synthetic vector, such as PEI, may have potential utility as prophylaxis in humans.

To determine the effectiveness of siRNA delivery in treating an ongoing infection, mice infected with 3000 pfu of PR8 were treated i.v. with 60 μg of PA- or NP-siRNA in complexes with PEI 5 h after infection. A 10-fold reduction in virus titer was observed in both cases. When 120 μg of NP-siRNA was used in complex with PEI, the virus titer dropped to undetectable levels, representing over a 1000-fold reduction. Significant (ca. 5-fold) reduction in virus titer was observed even when siRNA was administered 24 h after infection and the lungs were then collected 28 h later.

Independent and simultaneous studies by Tompkin et al. [57] involving the same siRNAs, but different doses, strains of mice and virus, as well as route of administration and carriers have obtained complementary results supporting the generality of this approach. The method of administration of siRNA employed by Thompkin et al. is rapid i.v. injection of a large volume of siRNA solution (known as hydrodynamic or high pressure transfection), and this method is rather traumatic and, hence, unlikely to be useful in humans compared with other routes of administration, such as intranasal, i.t. or i.v. injections of small volumes of therapeutic agent.

4.1.2. Efficiency and lung specificity of PEI-mediated delivery greatly depend upon the hydrolytic purity of the polycation

We reasoned that the effective prevention and treatment of influenza virus infection in humans by siRNAs requires not only safe and effective means of delivery but also delivery vectors that are affordable. The cost of jetPEI from the vendor is about $600 for 0.1 ml of a 300-mM solution. Thus for a human with a 50-kg body weight (i.e., 2500 times above that of a mouse), the amount of jetPEI required for a single injection of siRNA would cost tens of thousands of dollars. A far cheaper linear PEI ($25/g) of a similar molecular weight available from another commercial vendor (Polysciences Inc., Warrington, PA) exhibited markedly lower efficiency and specificity compared to jetPEI. This difference appeared intriguing, and therefore we decided to investigate the mechanism involved in detail. NMR analysis indicated some 11% residual acyl groups in the commercial linear 25-kDa PEI. We found that removal of these residual *N*-acyl moieties from the commercial polymer by simple acid hydrolysis at elevated temperature enhanced its plasmid DNA delivery efficiency 21 times *in vitro* and as much as 10,000 times in mice with a concomitant 1500-fold enhancement in lung specificity. These observations indicated that hydrolytic purity of PEI was important for both transfection efficiency and specificity. To confirm this hypothesis and to

reduce the costs still further, three additional linear PEIs of 22-, 87-, and 217-kDa molecular weights were synthesized *ab initio* by acid-catalyzed hydrolysis of 50-, 200-, and 500-kDa poly(2-ethyl-2-oxazoline)s (under $0.5/g, Polysciences or Sigma-Aldrich), respectively, yielding the pure polycations isolated as their hydrochloride salts. PEI87 and PEI217 exhibited the highest transfection efficiency *in vitro* – 115-fold and 6-fold above those of the commercial and deacylated PEI25s, respectively; moreover, PEI87 delivered DNA to the mouse lung as efficiently as the pure PEI25 but at a lower concentration and, importantly, with a 200-fold lung specificity.

These improvements stem from an increase in the number of PEI's protonatable nitrogens, which presumably results in a tighter condensation of plasmid DNA and a better endosomal escape of the PEI/DNA complexes as evident from DNA-binding experiments and acid-titration experiments respectively. Although the difference in the number of protonatable nitrogens between the fully deacylated PEI25 and its commercial counterpart is only 11%, it can have a profound effect on the polycation's behavior, as illustrated by the following calculation. Given the 43-Da molecular weight of the $–CH_2CH_2NH–$ monomeric unit, the average number of the monomers in PEI25 is 581; this number is also the length of the contiguous stretch of potentially protonatable nitrogens. Assuming a random distribution of the *N*-propionyl moieties in the commercial PEI25, its contiguous stretch of protonatable nitrogens is only 11. This 64-fold difference may substantially affect the stability of the polyplexes as they traverse through the various extracellular and intracellular barriers involved in systemic gene delivery. Systemic delivery in mice of the complexes of a siRNA against a model gene, firefly luciferase, and PEI25 and PEI87 afforded a 77% and 93%, respectively, suppression of the gene expression in the lungs. Furthermore, a polyplex of NP-siRNA and PEI87 resulted in some 94% drop of virus titers in the lungs of influenza-infected animals [34].

4.2. Respiratory syncytial virus infection

Respiratory syncytial virus (RSV) is the most common cause of serious respiratory infections (mostly bronchiolitis and pneumonia) in infants and young children, resulting in their hospitalization. Infants less than 6 weeks old, and children with underlying conditions such as premature birth, heart disease or lung disease, are much more likely to be hospitalized with RSV. RSV also causes cold-like symptoms and pneumonia in older children and adults (http://www.cdc.gov/ncidod/aip/research/rsv.html). The immunocompromised state of the high-risk population, the incomplete immunity developed even by natural RSV infection, and its short incubation period are the issues that complicate effective vaccine development against the infection.

RSV (a Pneumovirus) is an enveloped, non-segmented, negative-stranded RNA virus. Its genome of approximately 15,200 nucleotides is transcribed into 10 transcripts encoding 11 distinct proteins, including the two nonstructural proteins NS1 and NS2 expressed from separate mRNAs encoded by the first and second genes, respectively. Deletion of either NS1 or NS2 drastically attenuates RSV infection *in vivo* and *in vitro*, pointing to their important role in viral replication [58]. Furthermore, repeated RSV infections are common as a result of the incomplete immunity caused by natural infection, the basis of which is poorly understood. RSV infections were shown to be associated with a predominantly T helper type 2 (Th_2)-like response in infants [59]. Since infants experiencing RSV bronchiolitis are more likely to develop wheezing and asthma later in life, RSV is considered a predisposing factor for the development of allergic diseases and asthma. Recently two groups have independently employed siRNA-based approaches to inhibit RSV in mice, as outlined below.

4.2.1. Intranasal delivery of nanochitosan–siRNA NS1-encoding plasmid complex inhibits RSV in mice

Zhang et al. used a shRNA-based approach against RSV infection. Because bovine and human RSV NS1 seem to antagonize the type-1 IFN-mediated antiviral response [60,61] the authors reasoned that blocking NS gene expression might attenuate RSV replication and provide an effective antiviral and immune enhancement therapy against RSV. Thus, siRNA oligonucleotide sequences for RSV NS1, and siNS1 were cloned into the pSMWZ-1 vector [62]. Pre-transfection (complexed with the liposomal reagent Lipofectamine 2000) of A549 cells with siNS1 substantially reduced the expression of NS1 proteins, but not of other viral proteins, at 24 h after infection with a recombinant RSV expressing GFP (rgRSV). The level of expression of IFN-inducible genes in infected A549 cells treated with siNS1 was considerably altered, as shown by microarray data. Whereas A549 cells showed considerable siNS1-induced decreases in rgRSV-infected cell numbers and virus titers, no effect of siNS1 in Vero cells (type-1 IFN-deficient) was observed. Also, in parallel studies, Vero cells cotransfected with pEGFP and siEGFP, not siNS1, showed substantial knockdown (91.68%) of EGFP gene expression. These results showed a definitive role of siNS1 in the attenuation of RSV replication and implicate the type-1 IFN pathway in this process.

When siNS1 plasmid was administered as a complex (referred to as nano-siNS1) with the nanochitosan polymer Nanogene 042 (NG042) as a nasal drop 2 days before viral inoculation, the RSV infection and pulmonary pathology in BALB/c mice were substantially attenuated with concomitantly knocked-down expression of the RSV NS1 gene. In addition, the viral titer in supernatants of homogenized lungs was also significantly decreased in the siNS1-treated mice compared to controls. Mice treated with siNS1 displayed significantly lower airway hyper-reactivity (AHR) than untreated mice, as well as a considerable reduction in pulmonary inflammation, as evidenced by decreases in the goblet cell hyperplasia of the bronchi and in the number of infiltrating inflammatory cells in the interstitial regions compared to controls. Knockdown of the RSV NS1 gene significantly increased IFN-γ expression in the lung compared to controls. Examination of IFN-γ levels in the bronchoalveolar lavage fluid by ELISA revealed a 2-fold rise in concentration in siNS1-treated mice compared to control animals. Nano-siNS1 treatment also had a prophylactic effect. Mice were treated with the Nano-siNS1 2, 4 or 7 days before

viral inoculation. Analysis of viral titers 5 days after infection showed that the prophylactic effect of siNS1 can last for at least 4 days. To test whether prophylactic blocking of NS1 activity can induce anti-RSV immunity and provide protection from re-infection, mice were administered with the Nano-siNS1, inoculated with RSV (5×10^6 pfu/mouse) 2 days later, and then re-inoculated with RSV (1×10^7 pfu/mouse) after 16 days. Cellular immunity induced by RSV 5 days after infection was examined in these mice by intracellular cytokine staining of splenocytes for IFN-γ and IL-4. Splenocytes of mice treated with Nano-siNS1 showed an increase in IFN-γ production in both $CD4^+$ and $CD8^+$ T cells and also in IL-4 production in $CD4^+$ T cells compared with controls. Following secondary infection mice treated with Nano-siNS1 showed over a 1000-fold drop in the viral titers compared to control mice. Thus prophylaxis with siNS1 enhanced cellular immunity and attenuated the secondary RSV infection.

4.2.2. Intranasal delivery of naked P protein siRNA or its polycation/lipid complex inhibit RSV in mice

Bikto et al. [63] employed an siRNA against the P protein (phosphoprotein), an essential transcription factor of the L protein (large protein) of RSV, for inhibiting the virus in BALB/c mice. They administered siRNAs complexed with TransIT-TKO (a proprietary polycation/liposomal formulation from Mirus Corporation, Madison, WI) reagent intranasally and 4 h later challenged each animal with 10^7 pfu of RSV intranasally. The siRNA that was effective *in vitro* was also found to be highly effective *in vivo*. At a dose of 5 nmol of intranasal siRNA (averaging ~70 μg for double-stranded siRNAs) per mouse, the siRNA against the P protein mRNA (referred to as siRNA#1) slashed pulmonary RSV titers 99.98%. Notably, the siRNA alone, i.e., free of transfection reagents, also significantly reduced pulmonary viral titers. Finally, intranasal siRNAs with or without the Transit-TKO reagent caused no obvious discomfort in uninfected mice (as judged by normal coat, activity, appetite and weight gain, and lack of respiratory distress), suggesting a favorable pharmacology for drug development. Separately, intranasal *pretreatment* with 5 nmol (70 μg) of anti-RSV siRNA#1 complexed with TransIT-TKO abolished RSV infection. Again, siRNA administered without transfection reagent also showed substantial reduction of infection. The reagent-free siRNA was 70–80% as effective as siRNA complexed with TransIT-TKO.

SiRNA treatment also had a curative effect on already infected mice, which is an important goal in pediatric medicine. The RSV-infected mice maintained their body weight for about 4 days after infection, followed by a gradual loss that continued at least up to 9 days, confirming previous observations. In contrast, mice treated with siRNA before or at the same time as RSV continued to gain weight without interruption. Most mice receiving siRNA on day 1 were also difficult to distinguish from the sham-infected controls. Those receiving siRNA subsequently (days 2–4) showed gradually less and less protection, although substantial improvement of weight was still observed. In the RSV-infected mice, the viral titer rose until day 4–5, and then slowly dropped to undetectable levels by day 16. SiRNA treatment before or concomitantly with RSV infection slashed the titer by ~99.98% at all days tested. Administration of siRNA later in infection was progressively less effective, but the viral titer was generally lower than in the untreated controls on any day tested. These observations suggest that the RSV P siRNA had a curative effect when administered after infection and that the treated mice were always less sick and recovered more rapidly than their untreated cohorts.

These two studies involved two different RNAi methodologies (synthetic siRNA and plasmid-encoded shRNA) and mRNAs directed against two different proteins (P protein and NS1, respectively) of RSV. Interestingly both reports involved intranasal delivery of siRNA, either alone or as a complex with a polycation or a polycation/lipid co-formulation. Taken together, these two reports demonstrate that siRNA delivery to the lung is a viable therapeutic strategy against RSV infection.

It is worth noting that since the publication of these papers, a humanized monoclonal antibody to RSV, Palivizumab (Synagis®, http://www.medimmune.com), has become available. According to Medimmune, Synagis significantly reduces hospitalizations in the first 6 months in premature infants born at less than 35 weeks, infants less than 24 months of age with chronic lung disease and requiring treatment in last 6 months, and in children 24 months or younger with hemodynamically significant heart disease. A new anti-RSV antibody MEDI-524 (Numax, Medimmune Inc.) appears to be even more effective in animals and is undergoing clinical trials.

4.3. SARS: Intranasal delivery of a mixture of two naked siRNAs inhibits SARS in monkeys

Since SARS (severe acute respiratory syndrome) is a newly emerging disease, a safe and effective vaccine is not yet available, although some candidate vaccines have been advanced to monkey models and clinical testing. Individuals with SARS usually develop a high fever, followed by severe clinical symptoms including ARS with a diffuse alveolar damage (DAD) at autopsy, caused by SARS corona virus (SCV) [64,65]. The identification of SCV as the causative pathogen of SARS was achieved mainly by demonstration that exposure of cynomolgus macaques to SCV resulted in symptoms similar to those of individuals with SARS.

Several research groups have reported *in vitro* inhibition of SCV [66–68]. Li et al. [69] extended their studies into a Rhesus macaque (*Macaca mulatta*) model. siRNAs showing prominent prophylactic and therapeutic activity in cell culture, referred to as siSC2 and siSC5 (selected from 48 siRNA duplexes targeting the entire SCV genome), were evaluated *in vivo*, using initially a reporter gene assay in mice and subsequently a clinically acceptable intranasal administration in the recently established Rhesus macaque SARS model. The two siRNA duplexes, siSC2 and siSC5, target the SCV genome at spike protein-coding and ORF1b (NSP12) regions, respectively. Their targeted sequences are identical to strain TOR-2 used in the cell-culture study, to strain PUMC01 used in the macaque model, and to another 100 published SCV strains isolated during different phases of SCV evolution recently defined with

wide geographic distributions around the world. Moreover, these are the two most potent inhibitors for reducing SCV replication in FRhK-4 cells among a set of active siRNA duplexes. A synergistic anti-SCV activity was observed when a combination of siSC2 and siSC5 was applied in the cell-culture study showing the strongest prophylactic and therapeutic effects.

Li et al. first tested an equimolar mixture of siSC2 and siSC5 (referred to as siSC2–5), as well as two unrelated control siRNAs siCONc–d, in mice. The siRNAs were delivered intratracheally in 5% aqueous glucose or in INFASURF (Forest Laboratories, Inc., New York, NY). (INFASURF is a prescription pharmaceutical that is instilled into the lungs of newborn infants with, or at risk for, respiratory failure. It is a sterile, organic solvent extract of calf lung lavage, containing surfactant proteins B and C (SP-B and C), with a protein-B level closest to that of natural surfactant.) The siRNAs were co-delivered with pCI-scLuc plasmid. The pCI-scLuc plasmid contained corresponding siSC2 and siSC5-targeted DNA sequence between its cytomegalovirus promoter-driven transcriptional initiation site and luciferase encoding sequence. This setup allowed easy monitoring of delivery efficiency from Luc assay. Interestingly, co-delivery in 5% glucose resulted in a higher level of reporter gene expression and stronger RNAi effect than that delivered in the INFASURF solution. Hence the former (i.e., naked siRNA) was employed in 5% glucose for experiments in Rhesus macaque. With siRNAs instilled intranasally, observations of SARS-like symptoms, measurements of SCV RNA presence, and lung histopathology and immunohistochemistry consistently showed siRNA-mediated anti-SARS efficacy by either prophylactic or therapeutic regimens. The siRNAs used provided relief from SCV infection-induced fever, diminished SCV viral levels, and reduced acute diffuse alveoli damage. The 10–40 mg/kg accumulated dosages of siRNA showed no sign of siRNA-induced toxicity.

5. Conclusions and prospects

Several proof-of-principle experiments in mice and monkeys for the potential treatment of lung diseases in humans have shown encouraging results. Intranasal and intravenous routes involving naked siRNA, as well as its complexes with polycationic or liposomal vectors, have been tested. Unlike systemic delivery, naked siRNA delivery intranasally produces gene silencing comparable to that delivered with vectors. This is very significant given that the slow progress of gene therapy to the clinic has been due to the lack of safe and efficient vectors.

Alnylam Pharmaceuticals, a Massachusetts-based biotechnology company (http://www.alnylam.com/), recently completed two Phase I clinical trials with the siRNA ALN-RSV01 to evaluate its safety, tolerability, and pharmacokinetics in healthy adult volunteers. Both trials were double-blind, placebo-controlled, and randomized. In total, 101 human subjects were enrolled in the trials and 65 were exposed to ALN-RSV01. The subjects received a single or multiple daily doses of ALN RSV01, or a saline placebo, as a nasal spray. ALN RSV01 was found to be safe when administered in relevant doses, with a mild adverse event profile comparable to placebo's. There was no evidence of laboratory or electrocardiographic abnormalities in subjects exposed to the drug and, no significant systemic exposure to the ALN RSV01 that was administered intranasally. Nastech, a Seattle-based biotechnology company (http://www.nastech.com), is currently developing siRNA as influenza therapeutics. By targeting the conserved regions of the influenza viral genome, their siRNAs is expected to be effective against all known human and avian influenza strains. Alnylam also is developing RNAi therapeutics for pandemic flu, such as H5N1 [70].

Sirna Therapeutics, a San Francisco-based company (http://www.sirna.com) is exploring the potential for local delivery of siRNAs to the lung for the treatment of respiratory diseases including asthma, Chronic Obstructive Pulmonary Disease (COPD), and viral infections. They successfully demonstrated extended lung exposure (suitable for once a day dosing), with limited systemic exposure, following lung administration of formulated, chemically modified siRNAs. In the asthma program, potent and efficacious siRNAs have been developed which target the inflammatory cytokines responsible for driving the airway inflammation which underlies airway hyper responsiveness in patients with asthma.

In closing, the progress made in the area of siRNA-based strategies for the treatment of respiratory diseases in less than 5 years seems very promising, and it appears that siRNA therapeutics for lung diseases are on their fast track to the clinic.

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