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Review Article

Inhaled short interfering RNA as a promising approach to the therapy of viral respiratory infections

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Abstract

Respiratory viruses, exemplified by respiratory syncytial virus (RSV), influenza virus and parainfluenza virus (PIV), are major serial killers, claiming millions of human lives annually worldwide. Respiratory infection by RSV and PIV is the most prevalent cause of pediatric hospitalization in industrialized Western nations. In the U.S. alone, it leads to > 90,000 admissions per year at a cost of about $300 million. The lack of a reliable vaccine or drugs against these viruses, in part due to the high mutation rate of the viral RNA genomes, has led to a desperate search for novel strategies for intervention, the most recent of which is RNA interference (RNAi). Studies within the past 4 years have established RNAi as a reliable technology to silence viral gene expression via post-transcriptional mechanisms. In exciting recent results, intranasally applied short interfering RNA (siRNA) provided protection, as well as cure, against both RSV and PIV in a mouse model, supporting inhaled siRNA as a highly promising antiviral drug of the future. This review summarizes the delivery, function and pharmacology of inhaled siRNA.

Short primer on RNAi

RNA interference (RNAi) is a recently discovered, evolutionarily conserved intracellular pathway triggered by double-stranded RNA (dsRNA) (1, 2). RNAi works by a dice-and-slice mechanism in which long dsRNA is first cleaved by a specialized RNase, named Dicer, to generate 20-22-base-pair products with 2-nucleotide-long 3'-overhangs known as short interfering RNA, or siRNA (Fig. 1). The antisense strand of the siRNA is then recruited into the RNA-induced silencing complex (RISC), the functional component of which is the "slicer" RNase, Argonaute 2 (3). With this antisense strand as the guide, the RISC engages the complementary target mRNA, which is then sliced by Argonaute 2, thus achieving knockdown (Fig. 1).

Synthetic siRNA for acute infection

The use of RNAi as a tool really flourished after the demonstration that synthetic siRNA, when introduced into mammalian cells in culture, specifically destroyed complementary target mRNA, leading to silencing of gene expression (4, 5). As an alternative approach to siRNA, short hairpin RNAs (shRNAs, precursors of siRNAs) can be cloned and expressed from recombinant plasmids or viral vectors, which are then cleaved by intracellular enzymes to generate siRNA (5). The enormous potential of RNAi in basic research and therapy was immediately apparent when it was realized that siRNAs and shRNAs can be designed to destroy any unusual or aberrant mRNA that underlies a disorder. The past few years of the new millennium have witnessed successful testing against a variety of diseases and infections, including (6-12): diabetic retinopathy; age-related macular degeneration (AMD); Alzheimer's disease, Parkinson's disease and other neural disorders; autoimmune diseases; and essentially all major viral infections, including AIDS, hepatitis, respiratory syncytial virus (RSV), influenza virus and parainfluenza virus (PIV) infections.

The choice between synthetic siRNA and recombinant shRNA is dictated mainly by the nature of the targeted disease. In chronic disorders such as Alzheimer's disease and cancer, where continuous, long-term RNAi therapy is essential, recombinant expression of shRNA is preferred. However, recombinant expression has been the forte of gene therapy for years and is associated with

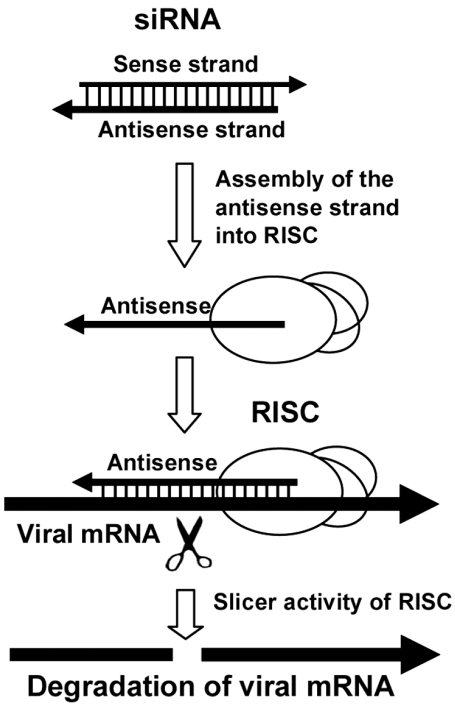

Fig. 1. The RNA interference (RNAi) pathway. Only the major steps relevant to recent therapeutics are shown in this highly simplified scheme. Essentially all double-stranded RNA (dsRNA) introduced in the cell for therapy is processed into small interfering RNA (siRNA), which has a roughly 20-base-pair double-stranded region and a 2-nucleotide-long 3'-overhang on each strand. The RNAi-induced silencing complex (RISC) formed with siRNA cleaves the complementary target RNA, such as a viral mRNA. The scissors indicate processing sites, and the arrowheads indicate 3'-ends of RNA strands.

adverse effects such as systemic infection by the virus, immune reaction against viruses and cancer from lentiviral vectors (13). We reason that as respiratory viruses produce only an acute infection lasting over a week or so (14), administration of synthetic siRNA over the short term is a better strategy due to its greater safety and the speed and ease with which siRNA can be made. The latter advantage is particularly critical for RNA viruses, which may rapidly mutate to become siRNA-resistant, as demonstrated for human immunodeficiency virus (HIV) (15). In the event of such mutations, the chemist can promptly design and synthesize a second siRNA targeting another sequence of the same virus for administration to the patient. This is not possible in recombinant delivery, as the cloning and growth of recombinant virus may take days and even weeks.

Viral infections of the respiratory tract

By virtue of its function, the upper and lower respiratory tract, including the lungs, is constantly exposed to airborne pollutants, particulate matter and pathogens. Influenza A and B viruses are significant respiratory pathogens in adult humans, particularly the elderly, with a healthcare tag of $1-6 million per million people in the U.S. alone. RSV is the most common worldwide cause of lower respiratory tract infections in the newborn and infants (14). PIV is second only to RSV in pediatric respiratory disease and infects a slightly older age group. In a recent WHO report, the global annual infection and mortality figures for RSV were estimated to be 64 million and 160,000, respectively (14). Commonly diagnosed as "croup", RSV disease manifests as pneumonia, bronchiolitis, rhinitis and otitis media, and a predisposition to asthma in later life. Premature infants and those with chronic lung disease or congenital heart disease are at particular risk. Although traditionally regarded as a pediatric pathogen, RSV can also cause life-threatening pulmonary disease in bone marrow transplant recipients and the elderly. Epidemics of all three viruses are particularly prevalent in the winter.

A number of features of this trio of respiratory viruses have contributed to difficulties regarding prevention and treatment (9, 14, 16). Firstly, their RNA genomes are highly mutatable, thus presenting a significant challenge in designing a reliable vaccine or antiviral therapy. Secondly, they are relatively difficult to grow in cell culture. Thirdly, a number of viral proteins bind to specific cellular proteins, adding to the difficulty of obtaining a cell-free viral material for vaccination. Finally, the immunopathology of all three is exceedingly complex, and this is particularly true of RSV, in which vaccine-enhanced disease, or "immunopotentiation", was first discovered. In short, RSV and PIV exemplify many intriguing challenges to an antiviral regimen.

Current treatment for viral respiratory infections

In a recent article, we covered the ups and downs of anti-RSV research and the lack of a reliable vaccine or antiviral agent (14). In brief, the main treatment available for RSV is a humanized monoclonal antibody against the RSV fusion (F) protein, palivizumab (Synagis™), marketed by MedImmune. The recommended dose is 15 mg/kg by i.m. injection, costing about $900 per infusion and requiring monthly injections throughout the RSV season. Prophylactic Synagis™ may be covered by insurance companies, but only if the physician feels that the child is at high risk. The most serious adverse reactions of Synagis™ are anaphylaxis and other acute hypersensitivity reactions, which are rare but may be under-reported. MedImmune also markets a live attenuated influenza vaccine, named FluMist®. Currently, there is no specific treatment for PIV infection. Finally, essentially all pharmaceutical companies have now canceled their RSV projects, although the continuing medical need and large market size clearly call for a safe and effective treatment regimen.

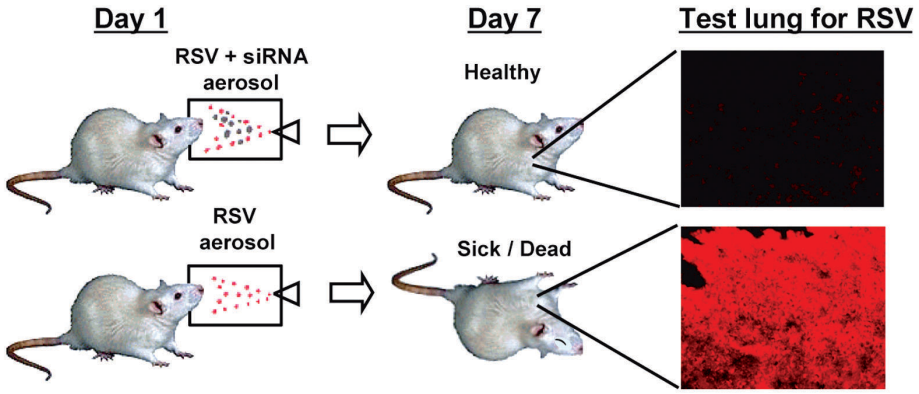

Fig. 2. Protection of BALB/c mice from respiratory syncytial virus (RSV) by inhaled siRNA. RSV (red dots), either alone or together with siRNA solution (black dots, siRNA#1 from Ref. 10), was sprayed as an aerosol in a closed breathing chamber designed for anesthetized mice. When examined a week later, mice receiving siRNA did not have pulmonary RSV infection or disease, in contrast to untreated mice, which were either very sick or died.

Inhaled siRNA against respiratory viruses

The landmark first experiment using synthetic siRNA as an antiviral therapy (7) was carried out shortly after the successful use of siRNA against cellular genes in tissue culture (4). In these studies, RSV growth was inhibited by targeting essential viral mRNAs coding for the viral transcription factor P (phosphoprotein) and viral F (fusion) protein (7). In both cases, specific ablation of the corresponding protein and mRNA was demonstrated. Long dsRNAs are known to activate an antiviral state by activating the dsRNA-dependent protein kinase PKR, thus triggering the interferon response. In contrast, synthetic siRNAs, due in part to their small size, do not activate the interferon pathway (4, 7, 10, 17, 18), and therefore function against specific viruses, thus bolstering their use in basic and clinical medicine.

This early success against RSV was soon validated against a number of viruses, including PIV and influenza, with siRNA or plasmid DNA expressing siRNA administered by the intravenous or intratracheal route (8). However, it was not until recently that direct intranasal delivery of siRNA was performed (10, 19). In one study, a complex of siRNA and chitosan nanoparticles was administered and afforded protection against RSV (19). The target was the viral NS1 protein, the exact role of which in viral growth or pathology was not addressed. Although nanoparticles in general are being considered as promising tools in drug delivery and medical imaging, their synthesis and application require specialized techniques and further optimization (20).

In a more straightforward study, synthetic siRNA against the RSV P mRNA was administered into mouse nostrils in two forms: carrier-free (naked siRNA dissolved in sterile water) or complexed with a transfection reagent (TransIT-TKO® reagent from Mirus Corp.). Both the siRNA and the subsequent challenge virus were equally divided and applied with a micropipette into the two nostrils (10). No special equipment was needed as the anesthetized animals inhaled all fluid through natural breathing. About 3.5 mg/kg of siRNA was considered the optimal dose. Upon challenge with RSV, nearly complete protection (> 90% survival) was noted. Similar protection was observed against PIV using specific anti-PIV siRNA. siRNA against PIV did not inhibit RSV, and *vice versa*. An exciting finding was that even siRNA without carrier offered protection when applied nasally, thus eliminating the possibility of carrier-related side effects or toxicity. Indeed, polyethyleneimine, a commonly used carrier for DNA and RNA (21), killed mice when applied nasally (10).

In a continuing effort to develop siRNA as an inhaled drug, we have recently reproduced these results by delivering siRNA by aerosol in closed chambers (Fig. 2). In both liquid and aerosol formulations, protection was demonstrated by a variety of clinical criteria. Firstly, very little virus was found in the lung tissue (Fig. 2), as examined by indirect immunohistochemistry and histopathology. Secondly, the treated mice maintained a normal low respiratory rate (10). Thirdly, leukotrienes, known to promote and predict for bronchoconstriction and to be elevated in RSV-infected children and asthma patients, were also elevated in the RSV-infected mice but not in the siRNA-treated infected animals (10). Thus, inhaled anti-P siRNA not only protected from RSV infection, but also prevented the development of respiratory symptoms characteristic of the infection. Inhaled siRNA did not trigger an interferon response (10, 17).

In another series of experiments, the curative potency of the siRNA was examined (10). The mice were first infected with RSV and then siRNA was nasally administered on different days thereafter. At all times tested, the siRNA prevented further growth of the virus and was associated with a better prognosis and more rapid recovery.

Multiple viral epidemics commonly occur during the winter. Inhalation of a mixture of two siRNAs against RSV

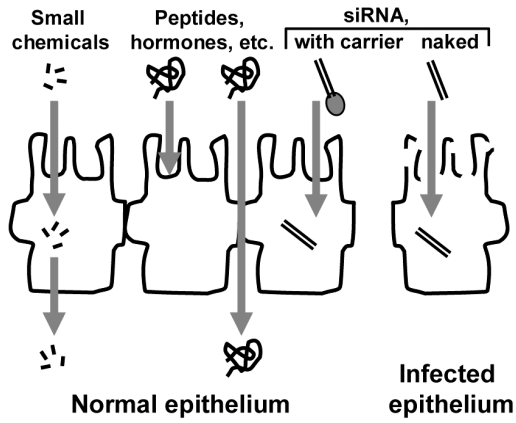

Fig. 3. Diagram of the intracellular transport of siRNA. In normal lung, only small molecules passively diffuse through the apical membrane of epithelial cells, whereas macromolecules may enter only through the tight junctions. Thus, siRNAs normally require lipid reagents as carriers for cellular uptake. We speculate that in virus-infected epithelia, the natural membrane barrier is lost and hence even naked siRNA can enter.

and PIV protected mice from joint viral challenge (10), indicating the possibility of multidrug inhalation therapy against multiple respiratory viruses. In short, 3.5 mg/kg of inhaled siRNA provided protection over about 3 days and substantial cure following respiratory virus infection. If necessary, another dose can be inhaled any time during the infection or as long as the risk of infection persists.

Inhaled siRNA drugs: design, pharmacology and pharmacokinetics

It is now recognized that the three main areas where improvement is needed to further develop siRNA for human use are specificity, stability and delivery. We will first discuss each parameter and then summarize how chemical modifications may affect them.

Target specificity is already a forte of siRNA because stringent base-pairing with the target mRNA is essential for the various steps of RNAi (Fig. 1). At very high concentrations, there is the possibility that siRNAs may activate the PKR pathway and have off-target effects. Thus, it is important to design a highly potent siRNA with an IC_{50} in the low nanomolar range.

Secondly, the stability of the drug in the body, delivery to the diseased tissue and permeation of the cell membrane are routine concerns in drug design. For siRNA, the stability problem is compounded by the nearly ubiquitous presence of RNases in all major body fluids, especially in blood. As shown in Figure 3, the delivery of small-molecule drugs generally occurs via free diffusion through the apical epithelial cell membranes. In contrast, larger molecules such as peptide hormones and antisense oligodeoxynucleotides (ODNs) are generally channeled via paracellular tight junctions or by carrier-mediated permeabilization of the lipid bilayer. The latter technique has traditionally been used for siRNAs, in which the siRNA is complexed with lipid-based transfection reagents such as Lipofectin™, Oligofectamine™ and TransIT-TKO™ (4, 7, 10), and introduced into cells or animals. Inhalation of naked siRNA obviates the use of such reagents.

Lastly, a variety of chemical modifications of siRNA were tested recently in an attempt to improve the parameters discussed above (12, 22). The major classes are summarized in Figure 4. The basic structure of an siRNA is shown at the center (compare with the scheme in Figure 1). In general, modifications at the 3'- or 5'-end of the sense strand, such as an amino linker, biotin and fluorescein, do not affect silencing activity. Thus, they may be used to link additional cell-permeable determinants or to trace the siRNA in the lung and the respiratory tract by fluorescent imaging. In contrast, the antisense

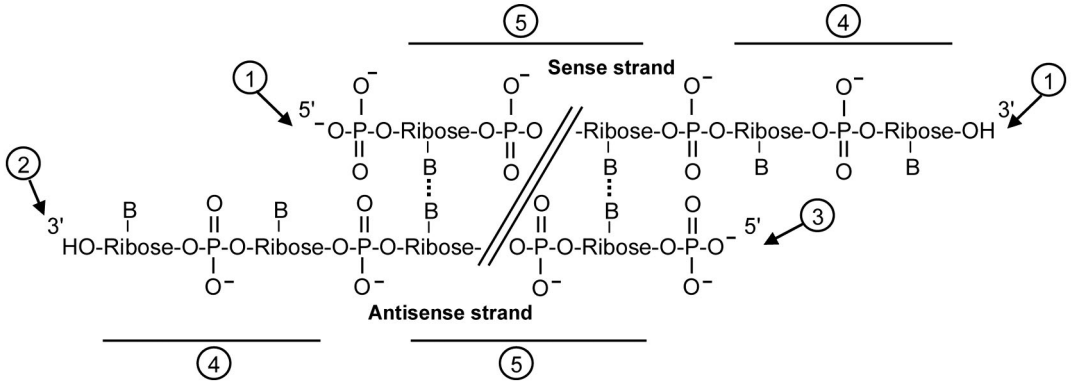

Fig. 4. Structure of an siRNA drug. A generalized structure of siRNA with the ribose sugars, phosphodiester bonds and bases (B) is shown. The molecular weight of an siRNA depends on the base composition and is approximately 14 kDa. The complementary bases within the base-paired region (broken in the middle to save space) are linked via two (AU) or three (GC) hydrogen bonds (dotted line). The exact bases depend on the target sequence. The circled numbers refer to chemical modifications discussed in detail in the text: ¿ terminal substitutions on the sense strand; À substitutions at the 3'-end of the antisense strand; Á substitutions at the 5'-end of the antisense strand; Â substitutions at ribose 2'-OH within the 3'-extensions; and Ã substitutions at ribose 2'-OH within the base-paired regions.

strand, which becomes a part of the functional RISC, is more selective. Whereas the addition of an amino linker and biotin at the 3'-end retains activity, fluorescein can be detrimental. The 5'-end of the antisense strand displays the opposite behavior: fluorescein is well tolerated, but an amino linker is not. The 2'-OH groups of the ribose rings have also been targeted for substitutions, and those within the 3'-overhangs, including 2'-deoxy, 2'-*O*-methyl and 2'-*O*-allyl, are tolerated well. In contrast, ribose modifications within the base-paired regions present a varied picture: a small number of 2'-*O*-methyl, 2'-fluoro-2'-deoxyuridine and 2'-fluoro-2'-deoxycytidine substitutions are tolerated, but exhaustive substitutions of all positions are detrimental for siRNA function. As these substitutions also improve stability, they are worth testing as inhaled siRNA. Any number of 2'-deoxy substitutions in the base-paired region seems to be detrimental. Internucleotide phosphorothioate substitutions (*i.e*., replacement of the phosphodiester P=O with P=S) make the RNA strands nuclease-resistant and enhance the stability of the siRNA; fortunately, the substituted siRNA also retains the silencing activity, although it may acquire cellular toxicity. These substitutions are particularly good candidates for testing in inhaled formulations. siRNAs containing a synthetic RNA-like nucleotide analogue, known as "locked nucleic acid" (LNA), are highly biostable and appear favorable for inhaled siRNA due to their enhanced activity.

Conclusions

Inhaled siRNA has the following advantages as a drug:

–It is a strong antiviral agent with both preventive and therapeutic value

–It can be designed and synthesized in hours, and new siRNAs can be tested as needed

–It can be applied in combination with other siRNAs in a multidrug regimen

–It is noninvasive

–It is easily administered via generic hand-held inhalers

–Dry siRNA in powder form is stable and can be shipped without refrigeration to remote areas and extreme climates; when needed, one can just add water, mix and inhale

–Many nasally applied drugs reach the central nervous system within minutes along both the olfactory and trigeminal neural pathways, thus bypassing the blood-brain barrier (23). If this is true for inhaled siRNA, it should provide an effective therapy for viral infections of the brain and the CNS, such as meningitis, encephalitis and myelitis, caused by a variety of viruses, such as echovirus, coxsackievirus, herpes, mumps, rabies, lymphocytic choriomeningitis virus and West Nile virus.

–siRNA dissolved in sterile water is effective when inhaled without transfection reagents, and chemical modifications may further enhance bioavailability and potency

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