Review

The promise, pitfalls and progress of RNA-interference-based antiviral therapy for respiratory viruses

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Advances in the understanding of RNA biological processing and control are leading to new concepts in human therapeutics with practical implications for many human diseases, including antiviral therapy of respiratory viruses. So-called ‘non-coding RNA’ exerts specific and profound functional control on regulation of protein production and indeed controls the expression of all genes through processes collectively known as RNA interference (RNAi). RNAi is a naturally occurring intracellular process that regulates gene expression through the silencing of specific messenger RNAs (mRNAs). Methods are being developed that allow the catalytic degradation of targeted mRNAs using specifically designed complementary small interfering RNAs (siRNAs). siRNAs are now being chemically modified and packaged into advanced delivery systems so as to acquire drug-like properties and the ability to deliver their effects systemically. Recent in vivo studies have provided proofs of the concept that RNAi may be useful therapeutically. Much of the design of these siRNAs can be accomplished bioinformatically, thus potentially expediting drug discovery and opening new avenues of therapy for many uncommon, orphan, or emerging diseases. Theoretically, any disease that can be ameliorated through knockdown of any endogenous or exogenous protein is a potential therapeutic target for RNAi-based therapeutics. Lung diseases in general are attractive targets for RNAi therapeutics, since the location of affected cells increases their accessibility to topical administration of siRNA, and respiratory viral infections are particularly attractive targets for RNAi-based drug discovery and development. RNAi therapeutics have been shown to exert potent antiviral effects against respiratory syncytial virus (RSV), parainfluenza, influenza, coronaviruses, measles and human metapneumoviruses in vitro and in vivo. Recently, a double-blind placebo-controlled clinical trial of an RNAi-based therapeutic against RSV demonstrated that this technology has therapeutic activity, representing the first proof-of-concept test of efficacy for RNAi's therapeutic effect in humans. This review discusses the science behind RNAi and the potential practical issues in applying this technology to various respiratory viral diseases.

Discovery, mechanism and perspective

Major advances that have changed the laboratory study of cellular functions are also altering our concept of how genes are finely controlled. The revolution in the understanding of RNA biological processing and control is now leading to new concepts in potential human therapeutics. The majority of RNA within cells is neither messenger RNA (mRNA), transfer RNA nor ribosomal RNA. This so-called ‘non-coding RNA’ exerts specific and profound functional control on regulation of protein production and, indeed, in the expression of all genes. These processes are collectively known as RNA interference (RNAi). Harnessing this naturally occurring RNA-mediated regulation of protein production has immense therapeutic potential, but has only now been developed enough to yield proof-of-concept human therapeutic data.

In October 2006, Andrew Fire and Craig Mello were awarded the Nobel Prize in Physiology and Medicine for their discovery and description of a naturally occurring cellular process known as RNAi in Caenorhabditis...
The ability to selectively silence specific gene function without affecting the genome itself has become a powerful research tool. RNAi is now arguably the most promising approach to gene silencing, and it has already proven to be effective in a variety of experimental systems. However, significant challenges remain before RNAi can be effectively translated into clinical applications. These challenges include the development of stable, efficient delivery systems, the optimization of therapeutic strategies, and the identification of specific targets for treatment.

One of the major challenges in the development of RNAi therapeutics is the delivery of RNAi molecules to target tissues or cells. Coronavirus RNAi therapeutics have been shown to be effective in vivo, but they have also been associated with immune stimulation and off-target effects. To address these issues, researchers have explored the use of antisense oligonucleotides (ASOs) and short interfering RNAs (siRNAs) as alternative therapeutic strategies.

The use of ASOs and siRNAs as therapeutic agents has been shown to be effective in several experimental models. For example, in vitro studies have demonstrated that ASOs can inhibit the expression of certain genes in a dose-dependent manner. Similarly, siRNAs have been shown to be effective in silencing specific genes in a variety of cellular and animal models. These findings suggest that RNAi therapeutics may have potential for the treatment of a wide range of diseases, including cancer, viral infections, and genetic disorders.

Despite these promising results, significant challenges remain before RNAi therapeutics can be effectively translated into clinical applications. These challenges include the development of stable, efficient delivery systems, the optimization of therapeutic strategies, and the identification of specific targets for treatment. Nevertheless, the potential of RNAi therapeutics as a new class of therapeutic agents is clearly apparent, and ongoing research is expected to continue to advance our understanding of this promising area of research.
siRNA can substantially reduce interferon stimulatory potential [3,12]. It has been argued that inducing interferon itself might not necessarily be a bad thing for an siRNA-based antiviral; indeed, some studies have observed non-specific immunostimulatory siRNAs to be effective antivirals [19]. However, traditionally, drug development has attempted to reduce interferon stimulation so as to avoid potential side effects.

Potential advantages of RNA-interference-based therapeutics including antivirals

Drugs affecting RNA targets offer several potential advantages over conventional small molecule drug development approaches. In many instances, it has simply been difficult to find small molecules that effectively inhibit many disease-specific proteins, even despite substantial

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Figure 1. Mechanism of RNA interference

Adapted by permission from MacMillan Publishers Ltd [3], copyright 2007. Two distinct and converging pathways of RNA interference (RNAi) are illustrated: the small interfering RNA (siRNA) and microRNA (miRNA) pathways. The siRNA pathway begins in the mammalian cell cytoplasm with cleavage of long double-stranded RNA (dsRNA) by the Dicer enzyme complex. This produces a specific 21 nucleotide RNA with 19 paired nucleotides and with two overlapping unpaired nucleotides at each 3′-end (Figure 2). These siRNAs are incorporated into the RNAi-induced silencing complex (RISC). If the siRNA loaded onto RISC has perfect internal sequence complementarity, an associated protein, Argonaut 2 (AGO2), cleaves the passenger (sense) strand, leaving the active (antisense) strand in the RISC. The siRNA antisense strand, remaining within the RISC, then recognizes target sequences of cytoplasmic messenger RNA (mRNA) in a sequence-specific manner, directing the RISC and AGO2 cleavage of this recognized mRNA. The siRNA remains within the RISC to direct multiple rounds of catalytic mRNA target cleavage. siRNAs can be synthesized and delivered to the cell cytoplasm at the point indicated by the dashed arrow. The cleavage of mRNA triggered either by a synthetic siRNA or an endogenously generated siRNA results in reduction of translation of the encoded protein. The final steps of protein down-modulation in the miRNA pathway involves direct translational inhibition and mRNA target degradation within processing (P)-bodies. These are different systems than are operative in the siRNA pathway.
screening efforts. Targeting protein production by way of reducing the presence of the mRNA from which they are translated, rather than targeting protein activity itself, expands the set of targets for drug treatment. A second advantage of RNAi therapeutics that is particularly important for viruses that cause acute disease is the ability to target genes with great sequence homology. Often the most variable proteins are those that are displayed on surfaces of cells and therefore are subject to immune recognition pressures. RNAi can instead target genes that produce essential viral proteins or replication intermediates, which are not surface proteins, and hence have less sequence diversity. A third potential advantage of an RNAi approach to antiviral drug development is the plasticity of RNAi-based drug design itself. RNAi-based drugs are designed largely in silico, and are based on knowledge of the genetic sequence of the target itself. Given the rapid sequencing technology currently employed, even newly discovered viruses and diverse strains are rapidly sequenced. Since the activity of an siRNA is based on its antisense strand sequence being complementary to the target, a knowledge of the sequence of the target can be used to rapidly design a large number of siRNAs. This can be performed on a personal computer. The siRNAs can then also be screened using easily accessible existing software, to eliminate sequences that are also represented within the sequenced human genome. The ratio of siRNA sequences that are complementary to a target and that also demonstrate knockdown activity for that target is approximately 1:4. This is several orders of magnitude greater than for conventional small molecule screening programmes, where often only a handful of active ‘hits’ are encountered in a screened library of approximately 10^6 chemical compounds. When one embarks upon screening programmes, fully one-tenth to one-third of complementary siRNAs have demonstrable activity against the target. Further screening can then, in principle, be accomplished by selecting siRNAs that are predicted to bind more tightly to RISC. Shortening the screening time, lowering its cost, and substantially increasing the chances of finding a molecule that is active in vitro drastically alters the net present value of a programme and shortens development timelines. This may allow for the ability to develop economically viable therapeutics for less common or rapidly emerging diseases, a category in which many acute viral diseases can be placed. In the future, if RNAi therapeutic side effects are generalizable to this particular new drug class, these side effects might be predictable, allowing even more cost-effective drug development.

Respiratory viruses as targets for RNA interference therapeutics

One of the problems remaining to be solved in developing an RNAi therapeutic is how to efficiently deliver the siRNA into the cells affected by a given disease. siRNAs that are not chemically modified are subjected to natural rapid degradation by ubiquitous endo- and exonucleases. Thus the half-life in the blood is short, with no detectable levels in the blood 10 min after administration [20]. Several methods are being developed to allow more efficient siRNA entry into the cytoplasm [21], and others to enhance its intracellular stability [3]. Delivery systems currently in preclinical and clinical development to overcome these hurdles will likely allow safe systemic delivery of siRNAs to humans. This topic has recently been reviewed [21]. However, as these technologies mature, it seems that direct topical application of the siRNA to affected cells may be a viable delivery
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RNA interference and antiviral therapy

method for human therapeutics. Topical delivery of antiviral siRNA has now been studied in several different viruses involving mucosal surfaces, including both HIV and herpes simplex virus [22–25]. In vivo models of topical delivery of RNAi-based therapeutics have demonstrated antiviral effects in animal models [26,27]. Similarly, topical delivery of an siRNA to the respiratory tract could be accomplished by inhalation as an aerosol. Respiratory epithelial cells of the lung have been shown to take up topically delivered siRNAs [28] and intracellular lung targets can be reduced in vivo with such topical administration (Figure 3). siRNAs are water soluble and robust enough to be easily aerosolized [29], with particle sizes (≤ 3 μm mass median aerodynamic diameter) which predict good deposition into both the upper and lower respiratory tracts. The delivery of RNAi-based drugs to the lung has been reviewed [30,31] and ≥ 1 siRNA utilizing this mode of delivery is in current clinical development [32–34].

The cellular distribution of respiratory virus infections such as respiratory syncytial virus (RSV), parainfluenza viruses, human metapneumoviruses and, to a lesser extent, influenza viruses allows for the possible application of an siRNA directly to the surface of infected or infectable cells. During active human infection, RSV and parainfluenza viruses almost exclusively infect ciliated respiratory epithelial cells [35–38]. In tissue sections of lung, even in immunocompromised individuals, the

Figure 3. Small interfering RNA delivered topically to the lung can reduce an exogenous protein

Adapted with permission from ©2004 The American Society for Biochemistry and Molecular Biology [28]. The figure shows western blots of the amount of two proteins in various tissues of mice after certain experimental conditions. Mice were subjected to ischaemia-reperfusion lung injury, which up-regulates expression of the exogenous protein, Heme oxygenase-1 (HO-1). Lungs of mice subjected to no such injury have little up-regulation of this protein. β-Tubulin is a housekeeping protein that maintains constant concentration throughout experimental conditions, and is therefore used as a quantitative control. Groups of mice were given different small interfering siRNAs topically to their lungs via nasal aspiration. No transfection reagents or vectors were used. Mice receiving the HO-1-specific siRNA show reduced expression of the targeted protein (HO-1). Mice receiving the sequence-mismatched siRNA (not targeting the HO-1 messenger RNA) show no effect on the amount of lung HO-1. The effect of the HO-1 siRNA was organ-specific and matched drug site of delivery. This represents, for the first time, that a topical delivery of an unaltered and unmodified siRNA was shown to reduce targeted protein expression.
infection is not seen to extend much beyond these superficial epithelial lining cells into deeper respiratory cells [37–39]. Furthermore, despite the challenge of delivering therapeutic aerosols to the upper and lower respiratory tract, topical (aerosol) delivery of antivirals targeting respiratory viruses have proven successful in concept by ribavirin (RSV) and zanamivir (influenza). Evidence that siRNAs enter respiratory epithelial cells in vivo after topical administration includes the finding that the parainfluenza virus loads can be reduced in vivo within the lungs of mice after treatment with siRNAs aspirated into their lungs [26]. This finding also helped to confirm the specificity and the mechanism of antiviral effect through which this viral reduction took place. siRNAs constructed specifically so that their sequence was mismatched to that of the respiratory viral target had no antiviral effect, whereas the siRNA matched to the sequence of the targeted RSV mRNA produced a profound effect after single topical administration [26].

Other factors contribute to the potential of respiratory viruses as excellent RNAi therapeutic targets. RNAi therapeutics can be selected to target viral genes or areas within viral genes that are known to have high levels of sequence conservation observed over both time and geographic area. Given the infidelity of viral RNA polymerase, such naturally occurring sequence conservation is likely due to selective disadvantage of mutations in these conserved regions. Such conserved regions can be identified and chosen as the specific RNAi therapeutic targets, which may help slow the development of viral escape mutations.

Viral resistance to RNA interference

The well-known history of the development of resistance to antivirals suggests that viral resistance to RNAi-based therapies would also be important. The fact that most of the important human respiratory viruses are RNA viruses, with intrinsically high mutation rates, adds to this concern. Because the fidelity of RNA-dependent RNA polymerase is poor due to lack of genome proofreading capability, the error rate during RNA genome replication is approximately $10^{-4}$–$10^{-5}$ mutations per nucleotide [40]. In a typical acute respiratory virus infection, such as RSV infection of infants, $10^7$ infectious units/ml are found in the respiratory secretions. This represents approximately $10^8$–$10^{10}$ copies of the genome [41]. Assuming the volume of the respiratory epithelial lining fluid in an infant, there are approximately $10^{12}$ viral genomes existing in a single individual at the peak of their infection. Mathematically, these $10^{12}$ genomes must have been the product of approximately at least $10^3$ replication cycles. Given that the length of most RNA viral genomes (including RSV) is approximately $10^4$ nucleotides, it is statistically likely that every possible single point mutation will have occurred multiple times and in combination by the time peak viral infection has occurred. This massive mutation-produced RNA virus diversity is subjected to the selection pressure exerted by the host immune response, or by an antiviral. RNAi-based therapeutics should exert a similar resistance-generating selection, as do traditional small molecule-based antivirals. So common and expected is this generation of antiviral resistance by RNA viruses, that the ability of new antivirals to select for resistant mutations is used as evidence of potent antiviral effect and even the mechanism of antiviral action.

Viral escape and development of resistance to RNAi in vitro has been studied extensively in HIV [42–46], and several other RNA viral infections, including HCV [47], morbilliviruses [48], hepatitis A virus [49], Japanese encephalitis virus [50] and poliovirus [51]. Because of the extensive potential for development of new human therapeutic agents utilizing RNAi, viral escape from RNAi is the subject of intense ongoing research. A comprehensive review of this topic is beyond the scope of this publication. The interested reader is referred to recent reviews on this topic [52].

Numerous different mechanisms of viral resistance to naturally existing RNAi have been identified [53], and many of these mechanisms have the potential to arise in response to exogenous siRNA antiviral therapies (Table 1). However, viral infections can be inhibited by RNAi-based therapeutics despite identified natural viral mechanisms of RNAi escape. Thus, the presence of these natural mechanisms identified in specific viruses does not necessarily dampen the potential for RNAi-based antivirals.

The most important mechanism of viral resistance generated in response to RNAi therapeutics is likely simple point mutation within the RNA target site. The most well-studied system evaluating RNA virus escape from RNAi is clearly HIV. However, the mechanisms
of viral escape from RNAi are likely different, and certainly of different importance, in chronic viral infections as opposed to acute viral infections, as is the case for respiratory viruses.

To evaluate the development of resistance to siRNAs, cell lines are stably transfected with short hairpin RNAs (shRNAs), which are exported from the nucleus and then are cleaved by Dicer into siRNAs in the cytoplasm. Using this system in HIV infection, RNAi reduced viral replication by 95%, but this antiviral effect was abrogated by day 25 because of a single nucleotide substitution in the target sequence [42]. Point mutations outside of the target region can also result in RNA antiviral escape (Table 1) [44], while nucleic acid substitutions within the target site do not always result in RNAi escape. Extensive study of siRNAs against HIV reverse transcriptase showed that complete escape from inhibition occurred with single substitutions in 10 of the 19 positions along the siRNA [45]. These studies and others [46] show that positions within the center of the siRNA produce RNAi escape. By contrast, mutations on either end of the siRNA (that is, positions 1, 2, 5, 18 and 19) remain inhibited by the siRNA. The basis for this positional mutational-tolerance effect is related to the known mechanism of siRNA incorporation into the RISC and its argonaut-protein-induced cleavage. Thus, knowledge of the natural sequence diversity of a virus, combined with knowledge of sites of permissible mutations can allow in silico selection of optimized therapeutic siRNAs.

The development of resistance to effective siRNAs has also been shown in the RNA viruses including hepatitis A [49] and C [47] and poliovirus. For poliovirus, siRNAs directed against essential targets initially showed efficient antiviral effect; however, resistant viruses emerged as early as 30–40 h in vitro. These escape mutations included silent point mutations (mutations not translating into an amino acid change) [51]. In these experiments, even G-U mismatch mutations conferred resistance. The observed ability of RNA viruses to escape RNAi through generation of silent single nucleotide mutations brings up the possibility that viral resistance to RNAi-based therapeutics might be generated more easily than to traditional small-molecule-based antivirals. Resistance dynamics to RNAi-based antivirals have been mathematically modelled and compared with traditional protein-targeting drug designs [54]. However, the vast expansion of the number of antiviral targets provided by RNAi-based therapeutics combined with the in silico ability to select these targets in extremely sequence-stable regions of the viral genome should mitigate this potential problem. Furthermore, the rapid development of resistance to an antiviral against an acute viral infection in human populations does not necessarily eliminate its therapeutic efficacy. The rapid development of influenza resistance to the adamantines (amantadine and rimantadine), even during a single treatment course, was demonstrated in early human clinical experience, yet these drugs continued to be effective for many years.

Several strategies have been proposed and even evaluated in vitro and in vivo to combat the problem of viral resistance to RNAi-based therapeutics (Table 2). Similar to the concept pioneered in HIV known as HAART, a cocktail of multiple siRNAs have been shown to be more effective than single siRNAs. With the knowledge of the potential escape mutations that are generated in vitro by sequencing viruses, which remain replication-competent whilst under siRNA antiviral pressure, one can theoretically design a convergent siRNA cocktail that anticipates and inhibits the preferred escape mutations and suppresses them. The success of this approach has been demonstrated recently in HIV [53,56] and to a limited extent in poxviruses [51].

RNA-interference-based therapies for influenza and other respiratory viruses

RNAi approaches to control other non-RSV respiratory viruses, including influenza, have received much attention recently, but rigorous, preclinical experimental data has either not been generated or remains unpublished to demonstrate that specific robust RNAi activity is responsible for the reported antiviral effects.

Respiratory viruses that have published RNAi-based therapeutic data now include measles virus [57], SARS coronavirus [58,59], Henipavirus [60], coxsackievirus and other enteroviruses [61,62], parainfluenza viruses [26], human metapneumovirus [63], and influenza [64–69] including H5N1 avian [70]. Many of these respiratory virus studies have been recently reviewed [33,71,72] and show both in vitro and in vivo antiviral effects, which are suggested to be due to RNAi. However, with the exception of RSV [73], the published literature has failed to adequately evaluate or control

Table 2. Mechanisms of avoiding or managing resistance to RNA-interference-based antivirals

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<th>Method</th>
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<tr>
<td>- Selection of viral targets with observed extreme sequence stability</td>
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<td>- Cocktails of siRNA (divergent sequences)</td>
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<tr>
<td>- Cocktails of shRNA (convergent, mutation-anticipating sequences)</td>
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<td>- Multiple siRNAs derived from stable transfection of single shRNA</td>
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<td>- siRNAs with both on-target sequence-specific RNAi-based antiviral</td>
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<td>activity and off-target interferon stimulatory properties</td>
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<td>- Targeting host cellular factors involved in viral pathogenesis</td>
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shRNA, long hairpin RNA; RNAi, RNA interference; siRNA, small interfering RNA.
for the potential off-target effect of proinflammatory stimulation as the cause of the observed effects.

As discussed in a previous section of this paper, double- and single-stranded RNAs are powerful, natural proinflammatory, stimulatory molecules. Their effect is mediated through the binding of TLKs 7 and 8, which mainly induce downstream interferon-α responses, and TLR 3, which mainly induces the interferon-γ pathway. The first exciting reports of anti-influenza activity [65,66] have now been questioned by Robbins et al. [74]. These investigators extensively re-tested the previously reported siRNAs, chemically modified these duplexes (2’-O-methylation) to ablate their proinflammatory potential, and used sensitive in vitro comparisons of interferon stimulation in capable cell lines to evaluate their proinflammatory potential. They report that the negative control siRNA duplex used commonly in many studies (an siRNA against green fluorescent protein) happens to have extremely low natural proinflammatory stimulating properties [74]. Therefore, compared with this green fluorescent protein siRNA, the studied siRNA duplexes (designed for sequence complementarity to various influenza essential mRNA sequences) have an observed antiviral effect. However, they report that 2’-O-methylation both ablates the siRNA interferon-stimulating properties and also completely negates the antiviral effect [74]. The effective proinflammatory antiviral effects of siRNAs against influenza have also been studied by other investigators [19].

Other frequent pitfalls causing misinterpretation of the therapeutic effect of siRNAs caused by immune stimulation include assays and sample collection times inadequate to detect immune stimulation. A more recent set of studies appears to confirm these off-target suspicions for influenza. Immunostimulatory motifs were shown to enhance antiviral effects in siRNAs targeting H5N1, and arguably little or no antiviral effect beyond that attributable to immune stimulation was observed [19,75]. Therefore, caution should be taken when reading the RNAi therapeutic literature and more rigorous techniques should be included in the future both to assess the existence of proinflammatory-induced off-target effects and to provide evidence for the presence of a specific RNAi mechanism. The latter is generally proven through identification and semi-quantification of the products of mRNA sequence-specific cleavage predicted by our knowledge of the activity of RISC. This has been shown to occur in vivo in RSV [73] and in endogenous gene targets [76].

Another RNAi-based approach to control of respiratory virus infections is the targeting of host pathways. This approach has the potential advantages of minimizing resistance due to the extremely low mutation rates of human genes and targeting common pathways that affect many viruses and emerging strains. Obvious disadvantages include the possible harm induced by interrupting cell pathways. An example of RNAi host gene knockdown affecting a human RNA virus infection has already been provided by HIV. The coreceptors of CCR5 and CXCR4, among others, have been studied as targets in vitro. A stably-transfected shRNA directed against CCR5 was given via haematopoietic stem cell transplantation to non-human primates [77]. These primates’ cells were less susceptible to simian immunodeficiency virus.

Recently, several similar papers were published using high throughput bioinformatic approaches employing large pools of siRNAs to selectively knockdown cellular targets for the purposes of bioinformatically illuminating key host pathways involved in human influenza infection [78–81]. These approaches uncovered multiple new host-cell pathways many of which could be targeted by RNAi-based therapeutics.

**Respiratory syncytial virus RNA-interference-based antiviral in clinical development**

The RNAi-based therapeutic that is furthest advanced in clinical development at this time is a siRNA targeting RSV. Because of the early work by Bitko and Barik [82], a major early thrust towards RNAi-based drug development was directed at respiratory viruses, and the virus chosen was RSV. Because of its relatively advanced state of clinical development, reviewing the siRNA therapeutic programme (ALN-RSV01) exemplifies the issues that might be encountered with other respiratory viral RNAi-based therapeutic programmes.

There is a major unmet medical need for an effective therapy for RSV infections in the otherwise healthy paediatric population, and in certain adult populations, including the frail elderly, those with chronic lung diseases (that is, chronic obstructive pulmonary disease and emphysema), those with immune deficiencies (especially haematopoietic stem cell and solid organ transplant recipients) or those with both chronic lung diseases and immune deficiencies (that is, lung transplant recipients). The only approved antiviral therapy for RSV (ribavirin) is rarely used in the paediatric population because of its potential teratogenicity and its limited effectiveness [83]. Ribavirin has never been adequately tested in any naturally RSV-infected adult population, and the single randomized trial in haematopoietic stem cell transplant recipients [84] was small in size and showed only trends toward antiviral and clinical benefits. Furthermore, there is no licensed RSV vaccine available. Prevention strategies for infants, relying on monoclonal antibodies, are partially effective [85], achieving approximately 60% reductions in RSV-related hospitalizations, are very costly, and are administered to <3% of the birth
P protein siRNA is limited by its specificity to one particular strain of RSV, while the effects of inhibition of the NS1 protein of RSV may be attributable to immune modulation, which results in the more robust clearance of the virus by the host rather than the direct targeting of the viral RNA. Furthermore, in both cases, definitive proof of an RNAi-mediated mechanism of antiviral activity remained to be established. Clinical development therefore initially focused on screening, identifying, and describing the pharmacology of a highly potent and specific human RSV siRNA with a broad spectrum of activity. Further effort was directed at definitive demonstration that the antiviral activity in vitro and in vivo was truly via an RNAi mechanism of action [73].

Consistent with their absence from the outer virus surface, the RNAs encoding the N, P and L proteins are among the most highly conserved regions of the RSV genome [87–89]. Therefore, a large number of siRNAs targeting the mRNAs of these genes were synthesized and screened through an RSV plaque reduction assay. Approximately 25% of the initial siRNA sequences showed good antiviral activity, and these candidate siRNAs were further screened in silico to avoid homology with the human genome [73]. This strategy, experimental data regarding lack of in vitro stimulation of tumour necrosis factor-α and interferon-α proinflammatory pathways in human peripheral blood mononuclear cells, and siRNA sequence-specific manufacturing preferences were used to select ALN-RSV01 for clinical development [73].

ALN-RSV01 is an unmodified naked siRNA (Figure 2) designed to inhibit the replication of RSV by interrupting the synthesis of the viral nucleocapsid protein (N protein). The sequence of the specific target is well-conserved throughout naturally occurring RSV A and B genotypes [73]. Delivering single doses of ALN-RSV01 topically to the cells of the respiratory tract by intranasal aspiration reduces RSV replication in mouse models of infection both prophylactically and therapeutically in a dose-dependent fashion [73] (Figure 4).

Clinical isolates chosen to broadly represent the known clades of RSV showed sequence homology to the siRNA target site and predictable in vitro susceptibility to ALN-RSV01. Furthermore, in an elegant series of in vivo experiments comparing ALN-RSV01, mismatched siRNAs and siRNAs with known low and high pro-inflammatory stimulatory activities, ALN-RSV01 was shown to exert its antiviral activity absent from any off-target proinflammatory effect. Furthermore, the products that the RSV N gene mRNA generated after RNAi-mediated cleavage were detectable in the ALN-RSV01-treated animals but not in the controls [73]. This series of experiments thus confirmed that the antiviral effect was mediated via the RNAi pathway itself.

RSV resistance to ALN-RSV01 has been studied in vitro. Up to eight rounds of RSV infection in the presence of cytoplastically transfected ALN-RSV01 was undertaken with subsequent deep (approximately ≤10%) cloning of recoverable virus and sequencing of the ALN-RSV01 target site. After eight rounds of RSV infection, no sequence-divergent viruses were detected [29]. However, stable transfection of siRNAs into the host cells (rather than intermittent exposure to transiently transfected siRNAs, such as ALN-RSV01) is required to exert sustained antiviral selection pressure and evaluate escape mutations. Thus, the laboratory techniques commonly used to identify RNAi escape mutations limit their direct applicability to predict such mutations occurring in response to exogenously applied siRNAs, such as ALN-RSV01.

ALN-RSV01 has been delivered topically to the nasopharynx, a histologically representative and easily sampled area of the human upper respiratory tract, to evaluate its safety and tolerability in healthy volunteers [20]. The Becton Dickinson nasal spray device (Franklin Lakes, NJ, USA), which is the same device used for administration of the live attenuated influenza vaccine, was used for dosing. This represented the first siRNA targeting a microbial pathogen to be tested in humans and was also the first siRNA to be administered to the human respiratory tract. There were no important differences in adverse events between ALN-RSV01 recipients and those receiving saline placebo. Based on this encouraging human safety data and previously conducted animal toxicology studies, the first randomized placebo controlled trial of an RNAi-based therapeutic was conducted to test the proof-of-concept that RNAi therapeutics can successfully silence targets in man [90].

This proof-of-concept study utilized the newly developed wild-type RSV human experimental infection model [91] with the good manufacturing practice (GMP)-produced purity and safety tested wild-type Memphis-37 strain (Meridian Life Sciences, Cincinnati, OH, USA). The study design was a randomized double-blind placebo-controlled clinical trial. Five daily doses of ALN-RSV01 (or saline placebo) nasal spray were administered with two daily doses prior to RSV challenge, and three daily doses post-RSV challenge. The pre-determined primary end point was reduction in the percentage of volunteers who became RSV-infected (as defined by multiple day
recovery of RSV by viral detection and quantification techniques including culture and quantitative RT-PCR). Of the 85 volunteers, 71.4% of the placebo recipients compared with only 44% of the ALN-RSV01 recipients shed RSV by culture ($P=0.009$). Trends in lower viral loads and symptom/disease measures were also seen favouring the ALN-RSV01 group. Careful measurement and logistic regression analysis of cytokine concentrations indicated that immune stimulation was not associated with the antiviral effect, but that exposure to the siRNA was independently and significantly ($P<0.05$) associated with the antiviral effect, thus validating the RNAi-based mechanism of action of the drug in humans.

This successful combined prophylactic/therapeutic proof-of-concept trial thus represented the first documented effect of an RNAi-based drug in humans [90] and prompted further clinical evaluation of ALN-RSV01 by aerosol in naturally infected adults. Lung transplant recipients acquire severe RSV infections that cause both acute pulmonary disease and also lead to progressive lung graft loss of function, pathologically defined as bronchiolitis obliterans syndrome (BOS) and progressing in grades to either death or requirements for re-transplantation [92–95]. A severity grading system of BOS is well-established. ALN-RSV01 was studied in a randomized, double-blind, placebo-controlled trial of 24 lung transplant patients experiencing acute infection with RSV [34,96]. Three daily doses of aerosolized ALN-RSV01 (versus saline placebo) were administered via the efficient eFlow nebulizer (PARI Pharma Gmbh, Starnberg, Germany). Ribavirin, intravenous immunoglobulin, palivizumab, corticosteroid use and BOS scores were roughly balanced between treatment groups at study onset.

ALN-RSV01 was well-tolerated, and there was no acute decrease in pulmonary function test results (forced expiratory volume within the first second) associated with aerosol dosing. Unfortunately, the randomization did not produce a balance in viral loads or in duration of symptoms prior to dosing, so that the observed lower viral loads in the ALN-RSV01 recipients could not be meaningfully attributed to effects of the drug. However, symptom scores were well-matched at baseline, and subsequent cumulative daily symptom scores were significantly lower in the ALN-RSV01 group ($P=0.035$). BOS occurring 90 days after dosing was significantly less in the
ALN-RSV01 group with 50% of placebo patients having new onset or progressive BOS at this pre-determined time point compared with only 6.3% of ALN-RSV01 patients (P=0.027). Because of these encouraging results, a larger similarly designed multinational Phase IIIb trial is now being conducted utilizing a longer 5-day dosing of ALN-RSV01 and longer (120-day) clinical evaluations. Overall, the clinical observations with topical ALN-RSV01 to date are encouraging and, because it is the RNAi therapeutic that is furthest advanced, help chart a path for future RNAi therapies to follow. Over the next several months to years, the promise of RNAi therapeutics will be even more rigorously evaluated, and respiratory diseases, especially respiratory viral diseases, are attractive early targets for clinical development using this novel class of therapeutics. Furthermore, the numerous advantages of RNAi may allow the development of therapies targeting epidemic and even emerging novel viruses.

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References


RNA interference and antiviral therapy


