

Therapeutic and Prophylactic Potential of Small Interfering RNAs against Severe Acute Respiratory Syndrome

Progress to Date

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Abstract

Severe acute respiratory syndrome (SARS), caused by the novel coronavirus SARS-CoV, produced a scare when it appeared in 2003 in China and later quickly spread to other countries around the world. Although it has since disappeared, its threat to human health remains. Therefore, studies on the prevention and treatment of SARS are important for dealing with epidemics of this and other infectious diseases. The most promising newly developed technology for intervention in SARS may be RNA interference, an endogenous cellular process for the inhibition of gene expression mediated by sequence-specific double-stranded RNAs. Numerous studies have reported the therapeutic potential of RNA interference for the treatment of various human diseases ranging from cancers to infectious diseases such as HIV and hepatitis. To date, most studies on inhibition of SARS-CoV replication using small interfering RNAs (siRNAs) have been conducted in cell lines *in vitro*. One study using siRNAs to inhibit SARS-CoV infection in Rhesus macaques demonstrated that siRNAs were effective both prophylactically and therapeutically with no adverse effects in the animals. Challenges remaining for the application of siRNA *in vivo* for SARS prevention and treatment include the specificity of the siRNAs and the efficiency of delivery. However, with improvements in siRNA design and delivery methods, RNA interference has the potential to become another major weapon for combating dangerous infections due to viruses such as SARS-CoV.

During the 2003–4 severe acute respiratory syndrome (SARS) epidemic,^[1] there was a high mortality rate^[2] and there were no rational methods for treatment. Empirical practices for treating the typical influenza-like symptoms were applied, such as using antibiotics, antiviral agents (ribavirin, oseltamivir, and HIV-protease inhibitors), corticosteroids, interferons, normal human immunoglobulin preparations, and Chinese herbs.^[3] Molecular diagnostic studies confirmed that a novel coronavirus, named SARS-CoV (the SARS-associated coronavirus), was responsible for the disease.^[4,5] This led to the search for rational regimens to treat the infection, including the generation of vaccines^[6–8] and the design of inhibitors of SARS-CoV replication.^[9] The most promising

newly developed technology for intervention in SARS may be RNA interference (RNAi).^[10]

SARS-CoV, as a member of the coronavirus family, is a single-stranded plus-sense RNA virus with a membrane envelope.^[11] The SARS-CoV genome is approximately 30kb in length and encodes a consensus leader sequence, the polyproteins 1a and 1b, and at least four major structural proteins including the N (nucleocapsid), S (spike), M (membrane), and E (small envelope) proteins. The four structural proteins constitute the virus particle. The N protein is a virion RNA-associated protein and forms a long and flexible helical nucleocapsid; this protein is also important for viral RNA synthesis. The S protein is a glycoprotein on the viral surface and is responsible for viral attachment and entry into host cells. The M

protein is necessary for virus budding and the E protein for virus assembly.

SARS-CoV replication requires first a partial duplication of its genome and the translation of some genome-encoded proteins. This leads to the first synthesis of a consensus leader sequence, a sub-genome, and the polyproteins 1a and 1b. The leader sequence is capped into the transcripts from the SARS-CoV sub-genome and plays a role in translation of the structural proteins.^[4] Polyproteins 1a and 1b are directly synthesized from the viral genome without the leader sequence. These proteins include papain-like cysteine protease (PLpro), 3C-like cysteine protease (3CLpro), and RNA-dependent RNA polymerase (RDRP), which are required for viral replication and transcription.^[4] Each step in the key processes during viral replication can be a potential target for the interruption of SARS-CoV replication. Since SARS-CoV replication also requires certain host proteins, genes from host cells involved in viral replication can also be selected as targets.

1. Small Interfering RNA (siRNA) and its Application

RNA interference is an endogenous cellular process for the inhibition of gene expression mediated by sequence-specific double-stranded RNAs (dsRNAs). This phenomenon was first observed in petunia flowers, and later reported in *Caenorhabditis elegans*.^[12] These dsRNAs, 21–23 bp in length, are produced by cleavage of a long RNA duplex by Dicer, a ribonuclease III-type enzyme. When produced, the small interfering RNA (siRNA) duplexes can activate a process of degradation of mRNA transcripts containing target sequences. This process is driven by the siRNA-enzyme complex or RNA-induced silencing complex (RISC) and results in inhibition of gene expression. Interestingly, when dsRNAs are artificially generated either by direct chemical synthesis or by folding single RNA into short dsRNAs with a hairpin loop, they can target the specific mRNA sequences and induce degradation of the mRNA. Therefore, RNA interference can be a tool for down-regulation of gene expression in cultured cells as well as in living organisms.^[13,14]

The two most important issues that must be considered before the application of siRNAs to down-regulation of gene expression becomes a reality are: (i) generation of effective dsRNAs; and (ii) delivery of the dsRNAs into targeted cells or organs. To address the first issue, researchers have established two strategies. The first one is to chemically synthesize siRNA.^[15] In this method, two single-stranded, complementary, short RNA oligonucleotides are synthesized. These two short oligonucleotides are then annealed into a dsRNA in a test tube. Usually short oligonucleotides (21–23 nucleotides) are designed with a two-nucleotide overhang to facilitate the activation of the RISC during the process of mRNA

cleavage. In addition, a 3'-hydroxyl group and a 5'-phosphate group are generated to enhance the function of the siRNA duplex.^[16] While it is simple and quick in its application, this chemical synthesis strategy has shown limitations in both stability and production cost of the siRNA.

To overcome the limitations of chemical synthesis of siRNA, a plasmid-based strategy was developed. In this strategy, a dsRNA of 21 bp with a hairpin loop of nine nucleotides is designed to be expressed from a plasmid under the control of a promoter.^[17] In cells, such RNA molecules are subjected to cleavage by Dicer to generate siRNAs. Since DNA plasmids can be easily and economically produced in *Escherichia coli*, this strategy has been widely adopted for the characterization of gene functions *in vitro* and for therapeutic studies.^[14]

To address the issue related to delivery of siRNAs into cells or living organisms, researchers have used several approaches, including: (i) direct introduction of the chemically synthesized siRNAs; (ii) employment of liposome-conjugated siRNAs or plasmids; and (iii) application of viral vectors to express dsRNAs with a hairpin loop. Each of the methods has both advantages and disadvantages. Recently, numerous papers have reported siRNA technology as a potent tool to knock-down gene expression or its therapeutic potential for treatment of various human diseases ranging from cancers^[18] to infectious diseases (e.g. HIV, hepatitis).^[14] In this review, we focus on the application of siRNA technology to SARS. Readers are also referred to another recent review on this subject.^[19]

2. Progress in the Utilization of siRNAs Against the Severe Acute Respiratory Syndrome-Associated Coronavirus

As the genomic sequence of SARS-CoV was determined and its mechanism of replication became apparent (similar to others in this coronavirus family), siRNAs targeting different genes of SARS-CoV were used by various groups to inhibit virus gene expression and thereafter to inhibit replication of the virus (see table I). The initial report came from two groups using the chemical synthesis strategy.^[20,21] The first group showed that two of six chemically synthesized siRNAs, based on the SARS-CoV genome sequence, resulted in the inhibition of viral cytopathic effect (CPE) in Vero-E6 cells infected with strain Y3-SARS-CoV.^[21] The cursory evaluation of the CPE of the transfected cells provided evidence that siRNA might be effective in inhibiting SARS-CoV replication. The second group demonstrated that three of six siRNAs designed to target the replicase A of SARS-CoV were moderately effective, whereas the other three were less effective.^[20]

Table 1. A summary of the small interfering RNAs designed to target sequences of the severe acute respiratory syndrome-associated coronavirus (SARS-CoV)

Target	Sequence	Method	Validation	References
Replicase 1A	5'-gcacuugucuaccuugaug-3'	Chemical synthesis	FRhK-4, GZ50 strain	20
Genome ^a	5'-ccaucaagccugugucguaTT-3' 5'-caucaaaauugcgcuaaagagTT-3'	Chemical synthesis	Strain Y3-SARS-CoV	21
Spike1	5'-gagacatatctaattgtgcc-3'	pBS/U6-Vector	Vero E6 cells, strain BJ01	22
Spike2	5'-gggctaccaccttatgtcc-3'			
RNA polymerase	5'-cttacctagctcgctc-3' 5'-gaatattaggcgaggctg-3'	pSUPER.retro	Vero E6 cells, strain HKU-39849	23
Leader	5'-ccaaccaacctcgatctc-3'	pBS/U6-Vector	Vero E6, BJ01	24
Spike	5'-aattgtcaatctcaagctc-3' 5'-aaagaacagcaagaagg-3'	Chemical synthesis	HEK293T, EGFP-S	25
RNA polymerase	5'-atcttaggattgcctacgc-3'	pSilencer1.0-U5	Vero E6, strain SARS CoV- p9	26
Spike	5'-gggctatcaacctatagatdTd-3'	Chemical synthesis	Vero E6, Hong Kong strain	27
Spike	5'-caaggcgattagctcaatdTd-3'			
3'-UTR	5'-cgtaactaaacagcacaagdTd-3'			
Leader	5'-gatggagagcctgttctt-3' 5'-cagccctatgtgtcatta-3' 5'-ctcactogtgagctcaatg-3'	pSilencer3.1-H1	Vero E6, strain BJ01	28
Nucleocapsid	5'-ggcatcgatgggtgcaact-3'	Cassette, pMD-18T	Mouse muscle, N-EGFP	29
Envelope	5'-ugaaggaguuccugauucuuTT-3'	Chemical synthesis	Vero E6,	30
Nucleocapsid	5'-agcuuaaacaacuccuggaacTT-3'		E-EGFP, N-EGFP, M-EGFP	
Membrane	5'-gauaauggaccccaucaaactTT-3'			
Genome	5'-gcuccuaauuacacucaacdTdT-3' 5'-ggaugaggaaggcauuuadTdT-3'	Chemical synthesis, C5W solution	Rhesus macaques, strain PUMC01	31,32
Spike	5'-cacugauuccguucgagauc-3'	Chemical synthesis, combination use	FRhK-4, Strain GZ50	33
Envelope	5'-cguuucggaagaacagguac-3'			
Nucleocapsid	5'-caagccucucucgucuccuc-3'			
Membrane	5'-ugcuugcugcugucucacag-3'			
Membrane	5'-guggcuuagcuacuucguug-3'			
Genome	5'-ggaugaggaaggcauuuTT-3'	Chemical synthesis, LNA modified	Luc reporter, HEK293, Strain Frankfurt 1	34
RdRP	5'-cugguacgauuucggugauTT-3' 5'-acugucuaaaccgguaauTT-3'			
Envelope	5'-aaggagtctgatctctgtgt-3'			
RdRP	5'-aaggacatgacctaccgtagac-3'	Vector (modified U6 promoter)	envE-pcDNA3.1/ RDRPpcDNA3.1, NIH3T3	35

a Sequence information was acquired from the authors.

LNA = locked nucleic acid.

Around at the same time, Zhang et al.^[22] used a vector-based strategy and designed two different siRNAs targeting the sequences encoding the S protein of SARS-CoV. These sequences were chosen because they were identical in different SARS-CoV strains. The authors inserted the siRNA sequences into the pBS/U6 vector with a U6 promoter, which expresses siRNAs in cells. This group of researchers was the first to demonstrate that the siRNAs inhibited S gene expression and virus replication in Vero E6 cells infected with SARS-CoV strain BJ01. This study provided strong evidence that siRNAs targeting the S gene could be employed as

potential tools to inhibit SARS-CoV replication. Even though the S protein has a high mutation rate,^[36] targeting its mRNA has also been reported by other groups to be effective in the inhibition of SARS-CoV replication.^[25,27]

Meanwhile, Wang et al.^[23] adopted a similar strategy by targeting the viral RNA polymerase gene of SARS-CoV. This group used a commercially available vector pSUPER.retro and designed six siRNAs. These researchers observed that in Vero E6 cells infected with the SARS-CoV strain HKU-39849, two of the six

siRNAs had a dramatic effect on the inhibition of viral replication. The effectiveness of targeting the RNA polymerase gene was further confirmed by Lu et al.^[26] and Meng et al.^[35]

To avoid the tedious work of constructing plasmid vectors to test effective target sequences, Zheng et al.^[31] chemically synthesized 48 siRNA duplexes targeting regions throughout the entire SARS-CoV RNA genome, including the open reading frames for several key proteins. They transfected these siRNA duplexes into fetal Rhesus kidney (FRhK-4) cells prior to, or after, infection with the virus, and evaluated the efficiency of the siRNAs on inhibition of viral replication. They found that four of the siRNA duplexes had a potent inhibitory effect on SARS-CoV infection and replication, evaluated by intracellular viral genome copy number and viral titers in the cell culture medium using quantitative reverse transcription PCR (Q-RT-PCR) and CPE-based titration, respectively. In this study, a prolonged prophylactic effect of siRNA duplexes with up to 90% inhibition that lasted for at least 72 hours was observed. Combination of active siRNA duplexes, targeting different regions of the viral genome, resulted in up to 80% inhibition *in vitro*.

Because they are convenient and rapid to generate, the chemically synthesized siRNA duplexes seem attractive for the identification of targets. Following the study by Zheng et al.,^[31] Shi et al.^[30] synthesized 26 siRNA duplexes and showed that three of these effectively inhibited the expression of a reporter gene carrying a target sequence in Vero E6 cells. To improve the effectiveness of inhibition, this group adopted a strategy utilizing a mutated nucleotide at the last pair of the duplex (with a TT overhang). They found this modification of the duplex increased the inhibitory effects on SARS-CoV gene expression. This observation confirmed that a mismatch in the 5' end of the antisense siRNA can enhance its effectiveness.^[37,38]

Recently, more siRNAs to inhibit SARS-CoV gene expression and replication have been reported. Wu et al.^[27] described three chemically synthesized siRNA duplexes targeting the S gene and the 3'-UTR sequences (si-SARS-S2, siSARS-S3, and siSARS-3'UTR2). Zhao et al.^[29] designed several siRNAs targeting the sequence encoding the N protein and, more recently, Meng et al.^[35] identified siRNAs targeting the E and RDRP genes.

Interestingly, two groups have reported the effects of targeting the leader sequence of SARS-CoV.^[24,28] Li et al.^[24] first identified the same leader sequence in the mRNA encoding different structural proteins. Based on this finding, they reasoned that siRNAs targeting the leader sequence should effectively inhibit viral replication in cell culture systems, since they would target a number of proteins simultaneously. This research group provided evidence that siRNA targeting the leader sequence, via a U6 promoter-based vector, had a stronger effect on the inhibition of SARS-CoV

replication than siRNAs targeting the S gene. Later, Ni et al.^[28] selected three of 32 potential siRNA sequences targeting the leader sequence. They demonstrated that three siRNAs delivered in the pSilencer3.0-H1 vector dramatically inhibited SARS-CoV replication as measured by either plaque formation or levels of mRNAs and proteins (S and N). While another group did not find any significant inhibitory effect of the chemically synthesized siRNA duplex targeting the leader sequence,^[27] Li et al.^[24] and Ni et al.^[28] have separately demonstrated the effectiveness of vector-driven siRNAs targeting the leader sequence. Whether there is a difference between the vector-driven and the chemically synthesized siRNAs remains unclear.

The rational design for effective siRNA inhibition of SARS-CoV replication remains difficult, as most authors have selected target sequences mainly by empirical testing. The selected target sequences usually have some specific features, as previous reports have suggested.^[14,39] For the ease of selection of siRNA target sequences, Meng et al.^[35] adopted a cell-based assay to screen for siRNAs to block SARS-CoV gene expression. This assay involved two genes encoding RDRP and the envelope E protein, respectively. These authors verified the published sequences and validated two siRNA sites for the RDRP gene and two sites for the envelope E gene. This assay provided a specific strategy to select effective siRNA sequences.

Considering that the virus may possibly escape from siRNA targeting through viral mutations,^[40,41] He et al.^[33] used a combination of siRNAs targeting different sequences. These authors reduced the dosage of the single siRNA duplexes to a minimal effective level and then tested the synergistic effect of the combination of the different duplexes. They found that the combination of siRNAs targeting two different sites had a significant synergistic effect on the inhibition of SARS-CoV replication in infected FRhK-4 cells. However, it is still unclear as to whether this synergistic role could overcome the problem of viral escape through mutation. In this study, they did not observe any synergistic effect of the combination of the siRNA duplexes at high dose, although this may be explained by a saturated dosage effect.^[20,31]

Currently, researchers have used both chemical synthesis and vector-based approaches to generate siRNAs for SARS-CoV. While the chemical synthesis approach is more rapid and easy to test, the RNA stability is problematic. To improve the biostability, Elmen et al.^[34] used a locked nucleic acid (LNA) approach for the design of siRNAs. As reported by Braasch et al.,^[15] LNA could increase the thermal stability of siRNA duplexes without any deleterious effect on their function. The study by Elmen et al.^[34] indicated that incorporation of LNA significantly enhanced siRNA stability and improved the efficiency of the unmodified siRNA on SARS-CoV inhibition. This study demonstrated that the modifica-

tion of the siRNA duplex could substantially improve its activity. Therefore, this approach may enhance the development of siRNAs as a therapeutic intervention for SARS-CoV.

To date, most studies on the inhibition of SARS-CoV replication using siRNAs have been conducted using cell lines *in vitro*. However, in the only animal study to date, Li et al.^[32] have reported their attempts at using siRNAs to inhibit SARS-CoV infection in Rhesus macaques. After initial screening, two chemically synthesized siRNAs were selected from 48 siRNAs based on the previous work of Zheng et al.^[31] The researchers treated the animals with the two siRNA duplexes in a commonly used glucose solution D5W prior to, and after, infection with SARS-CoV and found that all of the monkeys in the treated groups showed less severe symptoms than those in the control groups. They reported that during a 20-day period of observation after the administration of the siRNA duplexes, the treated monkeys showed an average body temperature of <38.9°C, while the control animals had an average body temperature of >39.1°C. The temperature changes were correlated with the changes in lung histopathology. The intriguing observation was that three of the four animals in each of the three siRNA-treated groups were free of SARS-CoV in the oropharyngeal swab specimens, based on measurements of SARS-CoV RNA copy numbers. This indicated that the SARS-CoV particles released from the animals' lungs were dramatically reduced by the injection of the siRNA duplexes. Whether siRNA inhibited SARS-CoV replication or blocked the spread of the virus within the lungs remains to be determined. However, this study clearly demonstrated that siRNAs were effective both prophylactically and therapeutically with no adverse effects in the animal study.^[10] The reduction of virus in the lungs may dramatically reduce spread to contacts, thereby being an effective approach to reducing spread of the virus between patients and caregivers.

3. Challenges to *In Vivo* Application of siRNA

Two challenges remain for the application of siRNA *in vivo* for SARS prevention and treatment. These involve the specificity of the siRNAs and the efficiency of their delivery.

3.1 Specificity and Possible Adverse Effects

The target specificity of siRNA is an important feature of this novel technology for therapeutic development. Highly specific siRNAs can be selected through screening to avoid potential off-target side effects. However, this high specificity can also be a problem since a single nucleotide change will diminish the effectiveness of the designed siRNA. Since mutations of the SARS-CoV genome can occur,^[36] which may result in viral escape from siRNA targeting,^[40,41] He et al.^[33] used a combination of different

siRNAs targeting different sequences. This had a further beneficial effect since it appears that the combination of the different siRNAs had a synergistic effect on the inhibition of SARS-CoV replication. Based on this data, it appears that the best approach would be to use a combination of siRNAs for maximal benefit; therefore, we suggest that highly effective siRNAs in combination should be used.

One possible adverse effect of *in vivo* application is that some siRNAs may trigger host immune responses such as the induction of interferon.^[42] However, since most siRNAs do not show this adverse effect, it is possible to conduct screening of siRNA sequences in animals before undertaking clinical studies. Another potential problem with the application of this technology *in vivo* is that when siRNAs are overexpressed, they may interfere with endogenous microRNA processing.^[43] This problem can be avoided by lowering the delivery dose or siRNA expression.

3.2 Delivery of siRNA

All the studies referred to in this review have demonstrated potent inhibitory effects of siRNAs (both chemically synthesized and vector based) on the replication of SARS-CoV in cultured cells or in animals. However, for clinical applications in both the prophylactic and therapeutic contexts, it is critical to improve the efficiency of siRNA delivery into specific human organs. As in the gene therapy field,^[44] siRNA targeting SARS-CoV is hampered by barriers to delivery. In the work done in Rhesus macaques, Li et al.^[32] adopted a very common glucose solution D5W to deliver the chemically synthesized siRNAs into the airways of animals. Although the experimental results demonstrated dramatic changes in SARS-CoV replication, it remained unclear how much of the siRNA duplexes had been successfully delivered into the airway epithelial cells of the animals, and how long the *in vivo* transfected siRNA duplexes were maintained in the cells. Clearly, this is an area where additional work is needed. However, we are confident that with all the recent work in gene targeting and vaccine formulation, this hurdle is not insurmountable.

Approaches for gene delivery to airways have been greatly improved over the past decade, especially those using viral vectors, such as the helper-dependent adenoviral vector (HDAd) which can transduce almost all the airway epithelial cells in mice and to a lesser degree in rabbits.^[45-47] Delivery of siRNAs with viral vectors should be more effective for prophylactic use, since expression of siRNAs from viral vectors is expected to last much longer than the duration of chemically synthesized siRNA in transfected cells. Although viral vectors can be used to deliver siRNAs for SARS-CoV, potential adverse effects, such as inflammation, have to be considered. In fact, siRNAs can be used to

down-regulate the expression of proinflammatory cytokines. For example, Cao et al.^[48] have shown that interleukin (IL)-8 expression can be down-regulated by an HDAd expressing an siRNA targeting IL-8 mRNA in airway epithelial cells. The concern of host immune responses to HDAd may be addressed by using anti-inflammatory drugs. In addition, adeno-associated viral vectors that do not cause host immune responses have been shown to be highly efficient in expressing siRNA in mouse livers^[43] and may be adapted to airway siRNA delivery as well. Therefore, these viral delivery approaches may be useful to translate RNAi technology into clinical applications.

To date, few studies have been performed to compare the efficiency of siRNA delivery *in vitro* with that *in vivo*; more animal studies will be helpful in screening for siRNAs with therapeutic potential. For the rational design of siRNAs for treatment of SARS, we can also learn from experience with the application of siRNAs to other diseases. In this regard, Schiffelers and Storm^[49] have reported a system named ICS-283, which was developed for systemic delivery of therapeutic siRNA to sites of pathological angiogenesis. In addition, Dykxhoorn and Lieberman^[50] recently commented on different approaches for the intravenous delivery of siRNAs in clinical applications. With the improvement of siRNA design and delivery methods, RNA interference will offer another major weapon for combating dangerous infections by viruses, such as SARS-CoV.

4. Conclusion

In this review, we have summarized the application of siRNAs to the prevention and treatment of SARS. Although there are still a number of problems that need to be overcome before this technology can be adopted in clinical applications, we believe that the progress in siRNA methodology will help us develop effective drugs to conquer this and other dangerous infectious diseases.

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