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# Development of interfering RNA agents to inhibit SARS-associated coronavirus infection and replication

## Introduction

In 2003, severe acute respiratory syndrome (SARS) spread to over 30 countries, infected over 8400 individuals and killed 813 people around the world. A novel SARS-associated coronavirus (SCoV) was identified as the causative agent.<sup>1</sup> It was likely that SCoV originated from wild animals and was later transmitted to humans as a SCoV-like viral pool, which was present in a large number of wild animals.<sup>2</sup> This syndrome can be transmitted through the airway or nasal passage, urine, water and domestic pets.<sup>3</sup> A vaccination or specific anti-SARS agent has not yet been developed.

The SCoV is a large, enveloped, positive-stranded RNA virus and its genome is composed of 30 000 nucleotides. The organisation of the genome is typical of the coronaviruses, following the characteristic gene order 5'-replicase (*rep*), spike (*S*), envelope (*E*), membrane (*M*), and nucleocapsid (*N*)-3'. The *rep* gene products are translated from genomic RNA and play key roles in viral replication and gene transcription, whereas the structural proteins are essential for virus package and infection. They are translated from subgenomic mRNAs, which are synthesised through a discontinuous transcription process.<sup>1</sup>

Small interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs) are potent agents for silencing gene expression, viral infection and replication in a sequence-specific manner. Replicase has long been the favourite target for antiviral drug development. We were the first group to demonstrate that siRNAs targeting the *rep* gene potently inhibited SCoV infection and replication.<sup>4</sup> However, recent studies revealed that viruses could easily escape siRNA targeting through fast mutagenesis.<sup>5</sup> Therefore, identification of multiple effective siRNAs targeting different sites or functional genes of SCoV could be an alternative treatment for any possible recurrence of the SARS epidemic. Our objectives were: (1) to design siRNAs to knock-down the four structural genes (*S*, *E*, *M*, *N*); (2) to test their antiviral effects; (3) to investigate the half-lives of siRNAs and dose responses; (4) to test the potential for combined synergistic antiviral effects of different siRNAs; (5) to test the potential synergistic effects of siRNA with interferon (IFN)- $\alpha$ ; and (6) to develop a deliverable recombinant adenovirus system (rAd-shRNA).

## Methods

This study was conducted from March 2005 to February 2007.

### siRNA design, synthesis, and screening

We designed and synthesised three siRNAs for each targeting gene. We selected the most effective siRNAs targeting each gene by cytopathic effect (CPE) assay. They were CACUGAUUCCGUUCGAGAUC (SARSi-S), CGUUUCGGAA GAAACAGGUAC (SARSi-E), CAAGCCUCUUCUCGCUCUC (SARSi-N), UGCUUGCUGCUGUCUACAG (SARSi-M1), and GUGGCUUAGCUACUUCGUUG (SARSi-M2). The sequences corresponded to nucleotides 23150-23169, 26113-26133, 28648-28667, 26576-26594, and 26652-26671 of GZ50 stain (GenBank accession number AY304495), respectively. SARSi-R or SRASi-4, the most potent siRNA targeting

## Key Messages

1. Small interfering RNAs (siRNAs) were designed to knock-down the four structural genes in severe acute respiratory syndrome-associated coronavirus (SCoV). Antiviral effects were confirmed by reduction of viral cytopathic effects and reduction in viral genomic RNA.
2. The siRNAs inhibited SCoV in a dose-dependent manner and had half-lives of approximately 24 hours.
3. The siRNAs acted synergistically with each other at low doses to enhance inhibition of SCoV. Specific siRNAs acted synergistically with interferon- $\alpha$  to massively (~100 000-fold) inhibit SCoV replication, compared to controls.
4. Recombinant adenovirus-based vectors expressing short hairpin RNA molecules were developed that potently inhibited SCoV replication and virus production.

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the *rep* gene (GCACUUGUCUACCUUGAUG)<sup>5</sup> was used as a positive control.

### **Cell culture, transfection and SCoV infection**

Foetal rhesus kidney (FRhk-4) cells were cultured and maintained in minimum essential medium (MEM) with 10% foetal bovine serum (FBS) [Invitrogen, CA, US]. Around 5000 cells were set in each well of a 96-well dish for viral infection and replication assay. The cells were transfected either without (negative control) or with siRNA at a standard concentration (200 nM) using OligoFectamine (Invitrogen, CA, US), in accordance with the manufacturer's instructions. Six hours after transfection, the culture medium was removed and the cells were washed twice with phosphate buffered saline before SCoV infection. One hundred microlitres of SARS-associated coronavirus (GZ50) diluted in MEM with 1% FBS was added to the transfected cells (multiplicity of infection 0.05). The CPEs were observed and recorded under phase-contrast microscopy 36 hours post-infection.

### **Construction of Adv-shRNA vectors**

We synthesised nucleotide oligos encoding effective siRNAs (SARSi-2, SARSi-3, SARSi-4, SARSi-S and SARSi-M1). A loop TTCG sequence was added between the sense and antisense strand and a transcriptional termination sequence TTTT was added after the antisense sequence. After annealing, the oligos were cloned downstream of a human U6 promoter (pAVU6+27). Then shRNA expression cassettes were subcloned into pAdeno-X (ClonTech, CA, US) to generate pAdeno-X-SARSi serial constructs. We packaged Adeno-X-SARSi viral vectors in HEK 293 Cells and purified the vectors using an adenovirus purification kit (ClonTech, CA, US) according to the manufacturer's instructions. FRhk-4 cells were transduced at a multiplicity of infection (MOI) 10:1 one hour before SCoV challenge.

### **Dual treatment with siRNA and IFN- $\alpha$**

To investigate the synergistic effects of siRNAs and IFN- $\alpha$ , siRNAs were first transfected into FRhk-4 cells. Four hours after transfection, the cells were treated with IFN- $\alpha$  at different concentrations. The cells were infected with SCoV 1 hour after incubation with IFN- $\alpha$ . The virus titre and RNA level of SCoV were measured as described above.

### **Quantitative reverse transcription-polymerase chain reaction**

We used real time (RT) reverse transcription-polymerase chain reaction (PCR) to quantify the intracellular viral RNA level as described previously.<sup>5</sup>

### **Titration of viral titres**

The conditioned medium from infected cells was subjected to 10-fold serial dilutions in MEM with 1% FBS and used for infecting cells according to the standard protocol. Briefly, cells were set in 96-well dishes 16 hours before infection. Seventy-two hours post-infection, CPE was

observed and recorded under phase-contrast microscopy, and the infectious viral titre was calculated.

## **Results**

### **Design and synthesis of siRNAs**

We designed and synthesised 12 siRNAs (3 for each gene) to target *S*, *E*, *M*, and *N* genes. We tested and selected the most effective siRNAs by CPE assay.

### **Determining antiviral effects of siRNAs**

#### **Protection of cytopathic effects**

FRhk-4 cells were set in 96-well dishes and transfected with or without siRNAs. The cells were infected with SCoV 6 hours after transfection, and CPE was monitored under phase-contrast microscopy. Cytopathic effects appeared when cells were transfected without or with GL2i (control siRNA targeting luciferase mRNA) and infected with SCoV. As effective siRNAs targeting replicase, cells transfected with effective siRNAs targeting structural genes (SARSi-S, SARSi-E, SARSi-M1, SARSi-M2 and SARSi-N) were protected from CPE.

#### **Reduction of viral genomic RNA copies**

The antiviral effects of individual effective siRNA were further characterised by determination of the copy number of intracellular viral genomic RNA using RT-PCR assays. The intracellular viral RNA level was reduced by 67.3 to 83.3% (83.3% by SARSi-S, 74.0% by SARSi-E, 77.5% by SARSi-M1, 81.7% by SARSi-M2, and 67.3% by SARSi-N) compared with the GL2i control at a standard concentration (200 nM). These results indicated that siRNAs potently inhibited SCoV RNA replication.

### **The half-lives of siRNAs and dose response against viral replication**

#### **Kinetics of intracellular viral genomic RNA**

The half-lives of siRNAs were investigated by determining viral kinetics of the cells being treated with siRNAs. The siRNA was transfected into FRhk-4 cells 6 hours before infection with SCoV. Quantitative RT-PCR experiments were carried out to determine SCoV genomic RNA copies in the infected cells at different time points (1, 6, 12, 18 and 24 hours post-infection). As the SCoV RNA level was markedly inhibited during the first 18 hours and then began to increase fast, the half-lives of siRNAs in FRhk-4 cells were about 24 hours in FRhk-4 cells.

#### **Inhibition of viral reproduction in a dose-dependent manner**

At doses of 1, 5, 20, 80, or 200 nM of SARSi-R in the transfection mixtures, the viral titre was reduced by 4.8, 16.6, 22.2, 25.4, and 33.4 folds respectively. Similarly, viral titres were reduced 3.1, 5.3, 10.5, 15.7 and 23.4 folds at doses of 1, 5, 20, 80, and 200 nM of SARSi-S, respectively. Similar results were obtained from SARSi-E, SARSi-M1 and SARSi-M2. SARSi-N showed lower relative activities at different dosages.

### ***Synergistic inhibitory effects of siRNAs combinations at low dose***

There was a saturated siRNA concentration, and combinations of siRNAs against the same gene did not increase the antiviral activities. If synergistic antiviral effects would be achieved with combined siRNAs targeting different genes at lower doses, it would offer an opportunity to develop cost-effective and specific agents to combat any SARS outbreak and drug resistance in the future. The concentration was decreased from 200 nM to 10 nM, the viral titre was reduced from about 30 folds to 5 folds by SASRi-R, 8 folds by SARSi-E, 2 folds by SARSi-S, -M1 or -M2, respectively. However, it was intriguing that anti-SCoV effects could be restored with two siRNAs targeting different genes at this dose. Compared with the control, the viral titres were reduced over 50-fold for SARSi-R/-S and SARSi-S/-E combinations, about 18-fold for SARSi-R/-M1 and SARSi-S/-M2 combinations, and over 30-fold for SARSi-R4/-E combinations, respectively.

### ***Synergistic effect of siRNA in combination with IFN- $\alpha$***

The viral titres were  $10^{6.5}$  particles/mL in the media of cells transfected with or without mock siRNA. When the cells were treated with IFN- $\alpha$  alone, the viral titres were reduced about 100 times (from  $10^{6.5}$  to  $10^{4.5}$  particles/mL). In contrast, the viral titres were reduced over 100 times by SARSi-M1 alone (from  $10^{6.5}$  to  $10^{4.25}$  particles/mL) and nearly 1000 times by SARSi-R alone (from  $10^{6.5}$  to  $10^{2.75}$  particles/mL). However, the viral titres were reduced 100 000 times when IFN $\alpha$  was combined with either SARSi-R or SARSi-M1. In conclusion, siRNAs exhibited synergistic antiviral effects with IFN- $\alpha$ . The viral RNA levels were significantly reduced and the viral titres were reduced more than 100 times the levels with either siRNA or IFN- $\alpha$  alone. This was particularly important, as it not only greatly enhanced antiviral effects, but also reduced costs.

### ***Inhibition of SCoV by recombinant Adv-shRNA vectors***

Adv-shRNAs potently inhibited SCoV replication and virus production. The intracellular viral RNA was reduced more than 95% (adv-SARSi-R) at MOI 10. The viral titres were reduced over 100-fold at low MOI (MOI=1), and further reduced over 1000-fold (but less than 10 000-fold) at higher MOI (MOI=10). Taken together, SCoV was potently inhibited by adv-shRNA vectors to at least a similar degree as the original siRNAs.

## **Discussion**

We hypothesised that RNAi targeting structural genes could also exhibit antiviral effects, which might show synergistic effects when combined with each other or with the effective siRNA targeting replicase gene.<sup>5</sup> This would reduce drug resistance generated via virus mutations.<sup>4</sup> In this study, we developed siRNAs targeting *S/E/M* structural genes, which inhibited viral RNA accumulation in host cells with lower efficiency while exhibited almost the same

inhibitory activities for viral reproductions. There may be several reasons for this phenomenon. Firstly, viral genomic RNA will be immediately translated into viral proteins and undergo a fast RNA replication phase when it enters cells. Replicase is encoded by viral genomic RNA and is directly responsible for viral RNA synthesis. Therefore, siRNA targeting the replicase region will directly reduce replicase. Secondly, the 3'-region of viral genomic RNA encodes for several subgenomic mRNAs. The subgenomic mRNAs are abundant in the host cells, which may reduce target efficiency or prolong the siRNA-induced cleavage of viral genomic RNA. Finally, active transcription and/or translation in the 3'-region of viral genomic RNA may block siRNA target sites. However, the structural gene products directly contribute to viral package and infectious activities. The accumulated infectious viruses rely on both replication and the correct, effective package. Therefore, SARSi-S/-E/-M1/-M2 could display a similar degree of inhibitory activities in the production of infectious virions as SARSi-R at saturation in the media.

Kinetics studies showed the intracellular viral RNA accumulation was potently inhibited in the first 18 hours, indicating that the transfected siRNAs in FRhk-4 cells had short half-lives (about 24 hours). Therefore, sustained shRNA expression vectors—Adv-shRNA vectors—were developed. These vectors showed similar antiviral effects as the original siRNAs, which would offer an alternative for anti-SARS therapy.

Various combinations of siRNAs targeting different genes produced synergistic anti-SCoV effects. A single siRNA at a low concentration (10 nM) significantly reduced antiviral activity while combination of two siRNAs targeting different functional genes restored their antiviral activities at saturated concentrations, suggesting that combinations of effective siRNAs could be used in clinical applications with reduced toxicity and at a lower cost. More importantly, the effective siRNAs tested in this study showed synergistic antiviral effects with IFN- $\alpha$ , which might provide a powerful anti-SARS tool in the future.

In conclusion, we developed multiple effective siRNAs targeting different genes of SCoV, including *replicase, spike, envelope, membrane and nucleocapsid*, which could potently inhibit SCoV replication in a dose-dependent manner. The half-lives of these siRNAs were about 24 hours in FRhk-4 cells and exhibited synergistic antiviral effects in combination even at very low doses. After conversion into shRNA by using adenoviral vectors, the respective shRNA exhibited similar antiviral effects as the original siRNA. These effective siRNAs also showed synergistic antiviral effects with IFN- $\alpha$ , which might provide a powerful anti-SARS tool in the future.

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