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Development of Interfering RNA Agents to Inhibit SARS Associated Coronavirus Infection and Replication

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Contents

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Summary:

SARS-associated coronavirus was identified as the etiological agent of Severe Acute Respiratory Syndrome (SARS) and a large virus pool was identified in wild animals. Virus generates drug resistance through fast mutagenesis and escapes antiviral treatment. However, no vaccine or specific antiviral treatment is available. We previously demonstrated that siRNAs against this novel SARS-associated coronavirus (SCoV) replicase inhibited virus replication. In this study, we aimed to develop cost-effective anti-SARS agents. Firstly, we developed siRNAs targeting viral structural proteins (i.e., spike, envelope, membrane, and nucleocapsid) that also inhibited viral reproduction. Secondly, we determined their antiviral kinetics and revealed that they exhibited synergistic effects when two siRNAs targeting different functional genes were combined. The potent antiviral effects could be achieved at a very low dose. Thirdly, we revealed that synergistic antiviral effects would be achieved by combination of interferon and siRNAs. Finally, we developed Adv-shRNA vectors and tested their antiviral effects. The results showed that SARS-coronavirus could be also inhibited by the vector expressed shRNAs. Our findings may pave the way for developing cost-effective siRNA agents for antiviral therapy in the future.

Introduction

Severe Acute Respiratory Syndrome (SARS) spread to over thirty countries and, infected over 8,400 individuals and killed 813 lives around the world in 2003 (www.who.int/csr/sars/en). A novel coronavirus (SARS-associated coronavirus, SCoV) was identified as the agent of SARS [1-6]. SCoV can produce a similar type of pneumonia in monkeys and other animals [7-10]. It was likely that SCoV originated from these infected wild animals and later transmitted to humans as a SCoV-like viral pool, which was present in a large number of wild animals [8-16]. SARS can be transmitted through the airway or nasal passage, urine, water and domestic pets [2, 9, 10, 17-19]. A vaccination or specific anti-SARS agent has not yet been developed.

SCoV is a large, enveloped, positive-stranded RNA virus and its genome is composed of 30-kb nucleotides [19, 20]. The organization 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' (Figure 1). The non-structural *rep* gene comprises 21-kb of the genome encoding two polyproteins (encoded by ORF1a and ORF1b) that undergo cotranslational proteolytic processing. The *rep* gene products are translated from genomic RNA, and play key roles in viral replication and viral gene transcription [19, 20]. These structural proteins are translated from subgenomic mRNAs, which are synthesized through a discontinuous transcription process [21-25]. The spike glycoprotein has been shown to be a viral ligand, which plays a critical role in virus binding to its receptor ACE2 or CD209L for viral entry into the host cells [26, 27]. Based on the peptide protection study, we showed that spike protein is a good target for prevention of viral infection [28, 29]. The nucleocapsid protein has been shown to play a role in viral genome package, transcriptional regulation of viral genes, and intracellular signaling [30]. The envelope protein and membrane protein are involved in viral package, viral secretion and virus-cell interactions.

Small interfering RNA (siRNA) and short hairpin RNA (shRNA) are potent agents for silencing gene expression, viral infection and replication in a sequence-specific manner [31-35]. Replicase has long been the favorite target for antiviral drug development. We were the first group to demonstrate that siRNAs targeting the *rep* gene potently inhibited SCoV infection and replication [36]. However, recent studies revealed that viruses could easily escape siRNA targeting through fast mutagenesis [37, 38]. Therefore, identification of multiple effective siRNAs

targeting different sites or functional genes of ScoV would be an alternative for the treatment of any future SARS outbreak.

Aims and Objectives:

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-life of siRNAs and dose response;
4. To test the potential combinational synergistic antiviral effects of different siRNAs;
5. To test the potential synergestic effects of siRNA with INF-alpha;
6. To develop deliverable recombinant adenovirus system (rAd-shRNA).

Materials and Methods

1. siRNA design, synthesis, and screening

siRNAs were rationally designed according to new strategies as described recently [31, 35]. The candidate siRNAs scored six or higher were selected and subjected to a BLAST search against GenBank to ensure that they were unique to SCoV genome sequences only. Three siRNAs targeting each gene were designed and chemically synthesized by Proligo BioTech Ltd (Paris, France). Their antiviral effects were detected by cytopathic effects (CPE) assay and those siRNAs markedly inhibited CPEs were chosen for this study. The sense-strand sequences of these siRNAs are CACUGAUUCCGUUCGAGAUC (SARSi-S); CGUUUCGGAAGAAACAGGUAC (SARSi-E); CAAGCCUCUUCUCGCUCCUC (SARSi-N); UGCUUGCUGCUGUCUACAG (SARSi-M1); and GUGGCUUAGCUACUUCGUUG (SARSi-M2). The sequences were corresponding to nucleotide 23150-23169, 26113-26133, 28648-28667, 26576-26594, and 26652-26671 of GZ50 stain (Accession number AY304495), respectively (Figure 1). SARSi-R, the most potent siRNA targeting *rep* gene (GCACUUGUCUACCUUGAUG, ref. 36), was used as a positive control in this study. A siRNA targeting luciferase mRNA [33], was used as a SARS-unrelated siRNA control. All the siRNAs were labeled with fluorescence at the 5'-end of the sense strands.

2. Cell Culture, transfection and SCoV infection

Fetal rhesus kidney (FRhk-4) cells were cultured and maintained in MEM medium with 10% fetal bovine serum (FBS, Invitrogene). Around 5,000 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), in accordance with the manufacturer's instructions. Six hours after transfection, the culture medium was removed and the cells were washed twice with PBS before SCoV infection. One hundred microliters of SARS-associated coronavirus (GZ50) diluted in MEM with 1% FBS was added to the transfected cells (multiplicity of infection 0.05). The cytopathic effects (CPE) were observed and recorded under phase-contrast microscope 36 hours post-infection [28, 36, 40]. The experiments were performed in triplicate and repeated at least three times.

3. Construction of Adv-shRNA vectors

We synthesized 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 TTTTT was added after the antisense sequence. After annealing, the oligos were cloned downstream of human U6 promoter (pAVU6+27). Then shRNA expression cassettes were released and subcloned into plasmid pShuttle to generated pShuttle-SARSi serial constructs. After recombination into plasmid pAdeno-X (ClonTech, CA), pAdeno-X-SARSi serial constructs were generated. We packaged Adeno-X-SARSi viral vectors in HEK 293 Cells and purified the vectors using an adenovirus purification kit (ConTech, CA) according to the manufacturer's instructions. FRhk-4 cells were transduced at a MOI 10:1 and 1:1 one hour before SCoV infection.

4. Dual treatment with siRNA and IFN-alpha

To investigate the synergistic effects of siRNAs and IFN-alpha, we first transfected siRNAs into FRhk-4 cells; four hours after transfection, we then treated the cells with IFN-alpha at different concentrations. Finally, we infected the cells with SARS-coronavirus one hour after incubation with IFN-alpha. The virus titers and RNA level of SARS-coronavirus were measured as described above.

5. Quantitative RT-PCR

Total intracellular RNA was isolated using RNeasy Mini kit (Qiagene, Germany) in accordance with the manufacturer's instructions. The reverse-transcription experiments were performed using ThermoScript RT-PCR systems (Invitrogene, CA). Real-time PCR was then performed using the forward primer 5'-GAAGGACCTACTACATGTGGGTACCTA-3' (GZ50 strain, nt 1303-1329), the reverse primer 5'-AACACTATGCTCAGGTCCAATCTCT-3' (nt 1401-1377) and the fluorescent probe 5'-(FAM)-CTAATGCTGTAGTGAAAATGCCATGTCCTGC-(TRMA)-3' (nt 1334-1364). The primers and probe bound the 5'-region of replicase 1A, that permitted us to measure the viral genomic RNA copies. Two microliters of the RT product (template) was used for each reaction. Forward and reverse primers (final concentration 900 nM) and the fluorescent probe (final concentration 250 nM) were mixed with Master Mix (ABI, USA) and real-time quantification was carried out using an ABI7900 Sequence Detection System. The PCR conditions were: 50 °C for 5 min, 95 °C for 10 min, then 40 cycles of 95 °C for 15 sec and 61 °C for 1 min [28, 41].

6. Titration of viral titers

The conditioned medium from infected cells was diluted at 10-fold serial in MEM with 1% FBS and used for infecting cells according to the standard protocol. Briefly, cells were set in 96-well dishes sixteen hours before infection. Seventy-two hours post-infection, CPE was observed and recorded under phase-contrast microscopy, and infectious viral titer was calculated [28, 36, 40, 41].

7. Instruments used in this study

PCR machine, real-time PCR machine, high speed centrifuge, Biosafety Level 3 (BL3) tissue culture system, fluorescent microscope, water bath, balance, pH meter, Agarose electrophoresis system.

Results

1. Design and synthesis of siRNAs

We designed and synthesized 12 siRNAs (3 for each gene) to target *S*, *E*, *M*, and *N* gene. We tested their effects by CPE assays (see below). We selected the most effective siRNAs (as shown in Figure 1) for the following studies.

2. Determining their antiviral effects

2a. Protection of Cytopathic effects (CPE)

Virus would easily escape antiviral treatment with a drug targeting a single site via fast mutagenesis [37, 38]. It is needed to develop multiple cost-effective and specific agents for clinical use in the future. Based on new rational design protocols [35, 39], we systematically designed and synthesized multiple siRNAs targeting each structural gene (*S*, *E*, *M*, and *N*) and detected their antiviral activities by CPE assay (described below).

FRhk-4 cells were set in 96-well dishes and transfected with or without siRNAs. The cells were infected with SCoV six hours after transfection, and CPE was monitored under phase-contrast microscopy. We recorded CPE at 36 hours post-infection using phase-contrast microscopy. The non-infected cells were healthy, and showed clear round shapes (Figure 2, panel I), while the infected cells displayed longer shapes, and some cells even floated away (Figure 2, panel II). No toxicity or CPE was discovered when cells were transfected with a siRNA (GL2i) targeting unrelated luciferase mRNA (Figure 2, panel III) or SCoV RNA without virus infection (data not shown). Cytopathic effects appeared when cells were transfected without or with GL2i and infected with SCoV (Figure 2, panel II and IV). As effective siRNA targeting replicase (Figure 2, panel V), cells transfected with effective siRNAs targeting structural genes (SARSi-S, SARSi-E, SARSi-M1, SARSi-M2 and SARSi-N, Figure 2, panal VI to X) were protected from CPE.

2b. Reduction of viral genomic RNA copies

We further characterized their antiviral effects of individual effective siRNA by determination of the copy number of intracellular viral genomic RNA using real-time PCR assays. We transfected the cells with siRNAs, and infected them with SCoV 6 hours after transfection. We then isolated the total cellular RNA from the infected cells 24 hours post-infection and quantified the viral genomic copies by real-time PCR. We found that 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 standard concentration (200 nM) (Figure 3). These results indicated that siRNAs potently inhibited SCoV RNA replication.

3. The half-lives of siRNAs and dose response against viral replication

3a. Kinetics of intracellular viral genomic RNA

We investigated the half-lives of siRNAs by determining viral kinetics after cells being treated with siRNAs. The intracellular RNA level is of course a key parameter for monitoring the kinetics of viral replication. Therefore, we carried out quantitative RT-PCR experiments to determine SCoV genomic RNA copies in the infected cells at different time points. At time point 1 hour post-infection, we found that the intracellular viral RNA copies were almost the same regardless of transfection with or without siRNAs, indicating that transfected siRNAs did not prevent virus entry. Therefore, we define the relative copy number as 1. In the control samples, the intracellular viral genomic RNA copies increased over 200 times within 5 hours, and increased another 25 times in the following 6 hours, as a result, the viral genomic RNA copies were amplified over 5,000-fold in FRhk-4 cells in the first 12 hours. However, the viral genomic RNA copies were only increased 2 fold either from time point 12 hours to 18 hours or from time point 18 hour to 24 hours (Table 1). These results suggested that the viral reproduction displayed two phases in FRhk-4 cells, i.e., fast replication phase (1-12 hour) and viral package and secretion phase (12-24 hour).

In the following 5 hours, the viral genomic RNA level was only reduced by 2 to 3 folds by all the tested siRNAs (table 1). In the next 6 hours (6~12 hours), the RNA copies were significantly reduced over 57 folds by SARSi-R. This strong inhibition was maintained until 18 hours and the viral genomic RNA copies were almost unchanged (24 hours post-transfection). Eighteen hours post-infection, the viral genomic RNA copies increased rapidly, and the inhibition was dropped to 13 folds at time point 24 hours (Table 1). For siRNA targeting *S* gene (SARSi-S), about five-fold inhibition was observed at time point 12 hours and 18 hours, but only about 3-fold inhibition was observed at time point 24 hours. For siRNAs targeting other structural genes, the viral genomic RNA copies were only reduced by 2 to 3 folds at all time points (Figure 4). Based on these data, we calculated that the half-lives of siRNAs in FRhk-4 cells were about 24 hours.

3b. Inhibition of viral reproduction in a dose-dependent manner

To elucidate the effects of siRNAs on viral titers (represents viral reproduction), we performed virus infectivity assay experiments. At 1 hour post-infection we removed the media and washed the cells twice with PBS containing 10 mM EDTA. Fresh MEM medium containing 1% FBS was then added to the cells, which were incubated for 24 hours. The viral titers in the conditioned media were measured by TCID50 assay. We found 200 nM of siRNAs markedly reduced infectious viruses which were secreted and accumulated in the culture media (data not shown).

To determine whether SCoV was inhibited by siRNAs in a dose-dependent manner, different amounts of SCoV-specific siRNAs were transfected into FRhk-4 cells before viral infection. In these experiments, 0, 1, 5, 20, 80, and 200 nM of SCoV specific siRNAs were used in the transfection reaction mixtures. GL2i was used as a carrier, to normalize transfection efficiency. The same dosage of total siRNAs (200 nM) was maintained in each transfection. The viral titers in the media were measured by TCID50 assay 24 hours post-infection. At doses of 1, 5, 20, 80, or 200 nM of SARSi-R in the transfection mixtures, the viral titer was reduced by 4.8, 16.6, 22.2, 25.4, and 33.4 folds respectively (Figure 5). Similarly, viral titers 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 (Figure 5).

4. Synergistic inhibitory effects of siRNAs combinations at low dose

It has been shown that there is a saturated siRNA concentration and combinations of siRNAs against the same gene would not increase the antiviral activities [32, 36]. An interesting question arose whether synergistic antiviral effects would be achieved with combined siRNAs targeting different genes at lower doses. If so, it would offer an opportunity to develop cost-effective and specific agents to combat SARS outbreak and drug resistance in the future.

To answer this question, we first reduced the concentration of siRNA from 200 nM to 50 nM in the transfection mixtures without carrier siRNA and observed their antiviral effects by TCID50 assays. Lower inhibitory effects were observed (data not shown). When the concentration was further decreased to 10 nM, the viral titer was reduced only about 5 folds by SASRi-R, 8 folds by SARSi-E, 2 folds by SARSi-S, -M1 or -M2, respectively (Figure 6).

It was intriguing whether anti-SCoV effects could be restored with two siRNAs targeting different genes at lower doses, because it would overcome the major cost barrier for clinic settings. To test this possibility, we transfected either a single siRNA or two siRNAs into FRhk-4 cells, and investigated their anti-SARS activities. In these experiments, the same total dosage of siRNAs (10 nM) was used for the transfection regardless of one or two siRNAs. We measured the viral titers in the conditioned media by TCID50 assay 24 hours post-infection. In experiments using a combination of two siRNAs, inhibition was significantly enhanced.

Compared with the control, the viral titers 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.

5. Synergistic effect of siRNA in combination with IFNα

We chose the most effective siRNAs (SARSi-R and SARSi-M1) for this study. We first transfected siRNAs into FRhk-4 cells at a final concentration 20 nM and 100 nM. Four hour after transfection, IFNα2 was added to into the culture media to the final concentration of 0, 0.125, 0.25, and 0.5 μg/ml. One hour later, the cells were challenged with ScoV. A siRNA targeting luciferase (GL-2i) and no siRNA transfected cells were used as control. As shown in table 2, the viral titles were $10^{6.5}$ particles/ml in the media of those cells tranfected without or with mock siRNA. When the cells were treated with IFNα alone, the viral titles were reduced about 100 times (from $10^{6.5}$ to $10^{4.5}$ particles/ml). On the other hand, the viral titers were reduced over 100 time by SARSi-M1 alone (from $10^{6.5}$ to $10^{4.25}$ particles/ml) and near 1,000 times by SARSi-R alone (from $10^{6.5}$ to $10^{2.75}$ particles/ml). However, the viral titers were reduced to 100,000 times when IFNα was combined with either SARSi-R or SARSi-M1. In conclusion, the siRNAs exhibited synergistic antiviral effects with interferon-alpha. The viral RNA levels were significantly reduced and the viral titers were reduced more than 100 times than either siRNA or IFNα alone. This is particular important, as it not only greatly enhanced antiviral effects, but also reduced cost.

6. Inhibition of SCoV by recombinant Adv-shRNA vectors

As shown in Table 3, Adv-shRNAs potently inhibited SCoV replication and virus production. The intracellular viral RNA was reduced over 95% even over 99% (adv-SARSi-R) at MOI 10. the viral titers were reduced over 100 folds at low MOI (MOI=1) while further reduced over 1,000 folds (but less than 10,000 folds) at higher MOI (MOI=10) . Compared with siRNA alone shown in Table 2, we showed that SCoV was potently inhibited by adv-shRNA vectors to at least a similar degree as the original siRNAs.

Discussion

The SARS-associated coronavirus is a novel identified RNA virus, which poses a severe threat to human health as there is a large SCoV-like virus pool in a number of animal species [1-18]. In this study, we aimed to develop effective RNA interference agents.

Specific siRNAs targeting SCoV genomic RNA would lead to viral RNA cleavage in the host cells. We previously identified effective siRNAs targeting *rep* gene which markedly reduced intracellular viral genomic RNA level [36]. We hypothesized that RNAi targeting structural genes could also exhibit antiviral effects which may show synergistic effects when they are combined. This would reduce drug resistance due to virus mutation. In this study, we developed siRNAs targeting *S*/*E*/*M* structural genes which inhibited viral RNA accumulation in host cells with lower efficiency (Figure 4) while exhibited almost the same inhibitory activities for viral reproductions (Figure 5). There may be several reasons for this phenomenon. First, viral genomic RNA will be immediately translated into viral proteins and undergo a fast RNA replication phase (as measured by time-point experiments) when it enters the cells. Replicase is encoded by viral genomic RNA and 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 several subgenomic mRNAs. The subgenomic mRNAs are abundant in the host cells [21-25], which may reduce target efficiency or prolongs the siRNA-induced cleavage of viral genomic RNA. Finally, active transcription and/or translation in the 3'-region of viral genomic RNA may block the 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 correct and 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 that RNA replication took place in two phases: a rapid replication phase (1 to 12 hours), and a slow replication phase (12 to 24 hours). In the first 12 hours, the viral genomic RNA increased near 6,000-fold. However, viral genomic RNA increased only 4- to 6-fold in the next 12 hours (Table 1). Viral genomic RNA replication was not potently inhibited in the first six hours post-infection in cells transfected with specific siRNAs targeting any of functional genes, for unknown reasons (Figure 4). Viral RNA accumulation was almost completely inhibited in cells transfected with SARSi-R until 18 hours post-infection. Viral RNA copies were also increasingly multiplied in cells transfected with other specific siRNA (2 to 5-fold) between 6 to 12 hours and between 12 to 18 hours post-infection. The copies of viral

genomic RNA increased faster in cells between 18 and 24 hours post-infection in some cases (SARSi-R, and -S). These results suggested that the transfected siRNAs in FRhk-4 cells had relatively short half-lives (about 24 hours). Therefore, a sustained expression of shRNA via viral vectors—Adv-shRNA vectors was 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 may produce synergistic anti-SCoV effects, as different genes play distinguishing functions in viral life cycles. We did not observe obvious synergistic anti-SCoV effects when two or three effective siRNAs targeted the replicase region [32]. In this study, we showed that specific siRNAs at low concentrations with highly concentrated carrier siRNA (total siRNAs maintained as 200 nM, Figure 5) exhibited potent anti-SCoV activity. As we know, DNA or RNA carrier reagents would increase transfection efficiency and extend the life of transfected DNA or RNA in the cells. Therefore, we first investigated whether siRNAs would display similar antiviral activities at lower doses without carrier siRNA. We found that a single siRNA at a low concentration (10 nM) without carrier siRNA significantly reduced antiviral activities while combination of two siRNAs targeting different functional genes displayed synergistic antiviral activities (Figure 6). Considering that SARSi-R, -S, -M1 and –M2 could only repress viral reproduction about 2- to 5-fold at 10 nM concentration, and combined siRNAs against two different genes exhibited over 18- to 50-fold reduction, these combinations were very effective. These results suggested that combinations of effective siRNAs targeting different genes could be used in clinical applications with reduced toxicity at a lower cost.

Our results demonstrated that siRNAs targeting function-distinguishing structural genes achieved varying degrees of success in inhibiting viral genomic RNA accumulation in host cells and reducing viral titers to almost the same level as the effective siRNA targeting replicase gene. In addition, we also showed that siRNAs targeting *rep*, *S*, *E*, and *M* genes at very low concentration displayed synergistic activities, as they restored even displayed better antiviral activities than a single siRNA alone at saturation. Our previous study showed that spike protein would be a good target for anti-SARS drug development and siRNA exhibited synergistic antiviral effects with chemical drugs [28, 35]. Taking together, we suggest that replicase, spike protein, envelope protein and membrane protein would be served as targets for siRNAs/shRNAs delivered with vectors (e.g., adenoviral vectors) for inhibition of viral replication and infections.

Conclusion

We developed multiple effective siRNAs targeting different genes of SCoV, including *replicase*, *spike, envelope, membrane and nucleocapsid*. They 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 at a very low dose. After converted into shRNA by using adenovirual vectors, the respective shRNA exhibited the similar antiviral effects as the original siRNA. More importantly, the effective siRNAs tested in this study showed synergistic antiviral effects with IFNα, which would provide a powerful anti-SARS tool in the future.

Implications

Our siRNAs, adv-shRNA vectors are powerful anti-SARS agents. They can be used either in individual or in combination, which would be further developed into clinical agents. The antival effects can be strongly enhanced by combination with interferon alpha.

Dissemination

We would fully support those companies to develop these siRNA/shRNA agents for clinical use if SARS recurs some day.

Publications

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Prof. KY Yuen: critical suggestion;

Prof. Joseph JY Sung: critical comments;

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Ms. Qi Dong: combined treatment with RNAi and IFN-alpha; construction of adv-shRNA vectors;
Ms. Pheobe Chow: Isolation of RNA;
Ms. Dan Du, constructs of Adv-shRNAs;
Mr. Cheng Lv, Isolation of RNA;
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Ms. Yan Ma, manuscript drafting (in part);
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Financial Statement

Table 1. Relative intracellular viral genomic RNA copy numbers (mean±sd) at different time points

	6 hour	12 hour	18 hour	24 hour
Control	228.4±3.9	5838.1±414.1	10680±782.3	29709.3±1628
SARSi-R	86.1±10.9	124.2±5.1	186.4±27.3	2159.8±88.9
SARSi-S	78.4±10.2	1196.4±56.1	1903±372.2	12152.2±2060.8
SARSi-E	84.2±16.8	2084.5±128.3	3012±65.3	12482.8±612.7
SARSi-M1	95.4±32.8	2256.9±551.8	3024.3±124.7	11337.6±834.5
SARSi-M2	104.6±5.1	2110.8±222.3	4263.5±243.1	8916.0±91.1
SARSi-N	110.8±10.9	2163.9±487.3	5618.4±144.9	14477.9±877.7

Note: The viral genomic RNA copy numbers at 1 hour post-infection was defined as 1.

Table 2: Synergistic effects of siRNA and IFNα (viral titers, ratio of viral RNA/GAPDH mRNA)

	IFNa concentration							
	0.5 ug/ml		0.125 ug/ml		0.125 ug/ml		0 ug/ml	
siRNA	Viral titer	Viral RNA level	Viral titer	Viral RNA level	Viral titer	Viral RNA level	Viral titer	Viral RNA level
SARSi-R 100 nM	$10^{1.25}$	0.218	$10^{2.25}$	1.33	$10^{2.75}$	4.66	$10^{2.75}$	2.81
SARSi-R 20 nM	$10^{1.75}$	0.648	$10^{2.75}$	13.8	$10^{3.25}$	38.5	$10^{4.75}$	149
SARSi-M1 100 nM	$10^{1.25}$	0.33	$10^{2.5}$	1.02	$10^{3.25}$	1.83	$10^{4.25}$	8.9
SARSi-M1 20 nM	$10^{1.75}$	2.7	$10^{2.5}$	8.89	$10^{3.75}$	20.5	$10^{5.75}$	92
GL2i 20 nM	$10^{4.5}$	8.15	$10^{4.75}$	50.5	$10^{5.75}$	159	$10^{6.5}$	268
Mock (PBS)	$10^{4.5}$	8.08	$10^{4.5}$	52.3	$10^{5.75}$	167	$10^{6.5}$	257

Table 3: The antiviral effects of Adv-shRNAs (viral titers, ratio of viral RNA/GAPDH mRNA)

	MOI					
	10:1		1:1		0	
shRNA	Viral titer	Viral RNA level	Viral titer	Viral RNA level	Viral titer	Viral RNA level
Adv-SARSi-2	$10^{2.75}$	11.3	$10^{4.75}$	53.2		
Adv-SARSi-3	$10^{3.25}$	29.3	$10^{5.25}$	58.3		
Adv-SARSi-4 (R)	$10^{2.25}$	2.4	$10^{4.5}$	49.1		
Adv-SARSi-E	$10^{2.75}$	8.1	$10^{4.5}$	51.2		
Adv-SARSi-M1	$10^{3.25}$	35.2	$10^{4.75}$	55.8		
Adv-GL2i	$10^{6.5}$	273	$10^{6.75}$	305	$10^{7.0}$	298

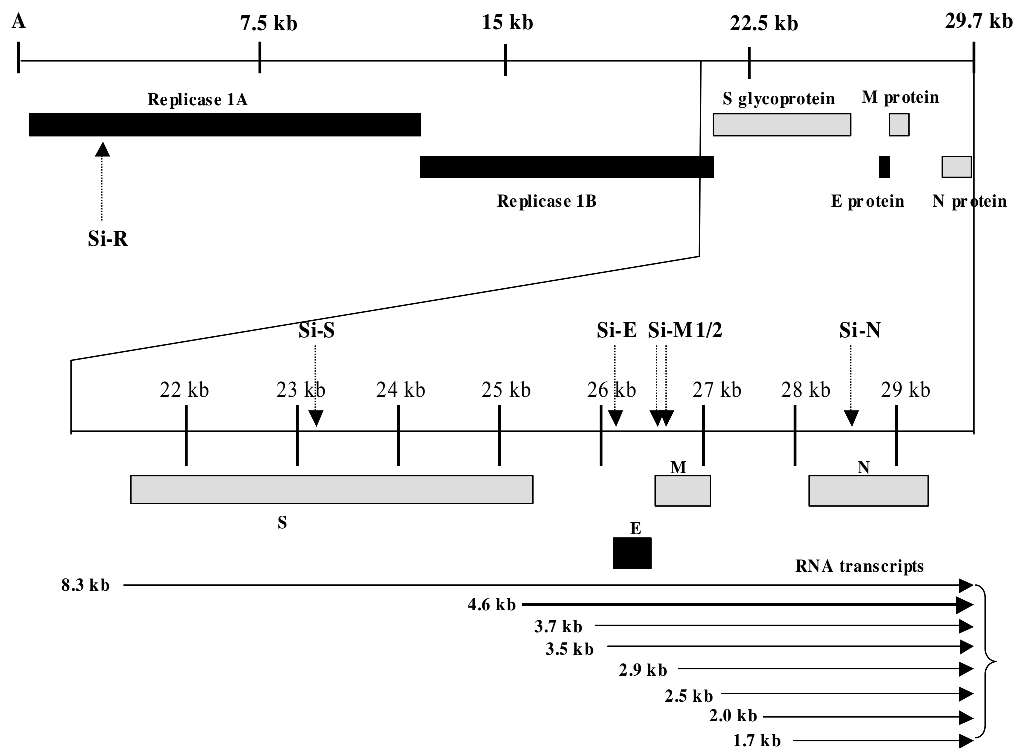

Figure 1. The diagram of the effective siRNA's targeting sites. SCoV genomic RNA is composed of 30 kb nucleotides. The replicase gene, which comprises about 60% of the genome, encodes two polyproteins that undergo cotranslational proteolytic processing. The downstream sequence of the replicase gene encodes four structural proteins (spike, envelope, membrane and nucleocapsid) and multiple potential nonstructure proteins (not shown). The SCoV replicase gene products are directly translated from genomic RNA, while the remaining viral proteins are translated from subgenomic transcripts. The target sites of the effective siRNAs are shown by arrows. Si- = SARSi-.

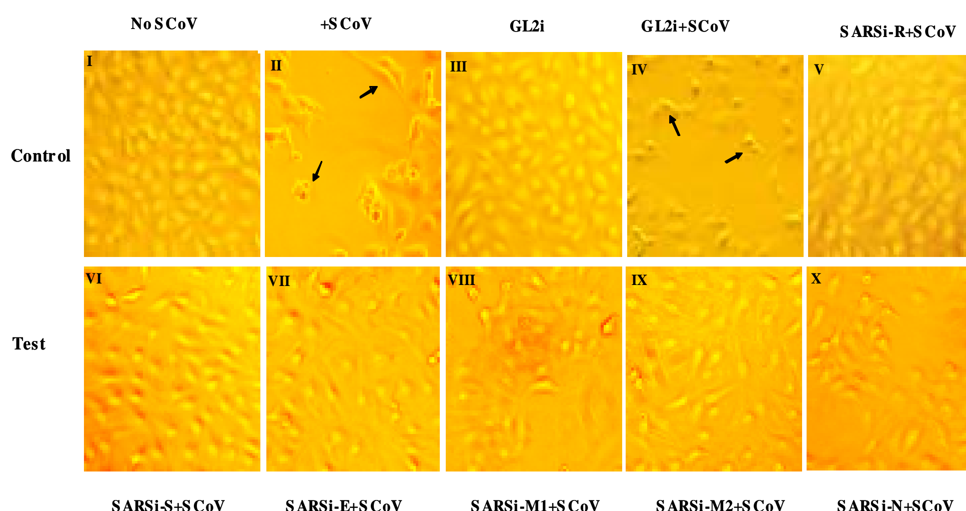

Figure 2. Inhibition of CPE by siRNAs. Cytopathic effects: FRhk-4 cells were transfected with (III to VIII, 200 nM) or without (I and II) siRNAs and infected with SCoV (II and IV to VIII). The photos were taken under phase-contrast microscope at 36 hours post-infection. The arrows show cytopathic cells.

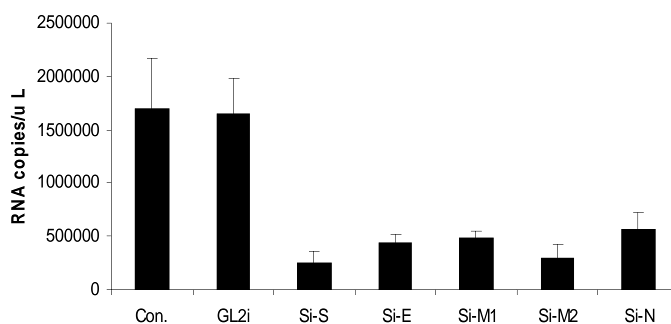

Figure 3. Reduction of intracellular viral genomic RNA copies by siRNA. The cellular RNA was isolated and quantitative RT-PCR experiments were conducted 24 hours post-infection. The experiments were performed in triplicate and repeated at least three times. Detection revealed that these siRNAs reduced viral replication effectively (student t test, $P<0.01$). The values (mean+/-standard error) were shown in a typical experiment. The values are: GL2i, $1.69x10^6±4.7x10^3$; SARSi-R, $1.2x10^5±5.5x10^2$; SARSi-S, $2.5x10^5±1.0x10^5$; SARSi-E, $4.4x10^5±1.0x10^5$; SARSi-M1, $3.8x10^5±2.2x10^5$; SARSi-M2, $3.1x10^5±1.2x10^5$; and SARSi-N, $5.7x10^5±1.5x10^5$.

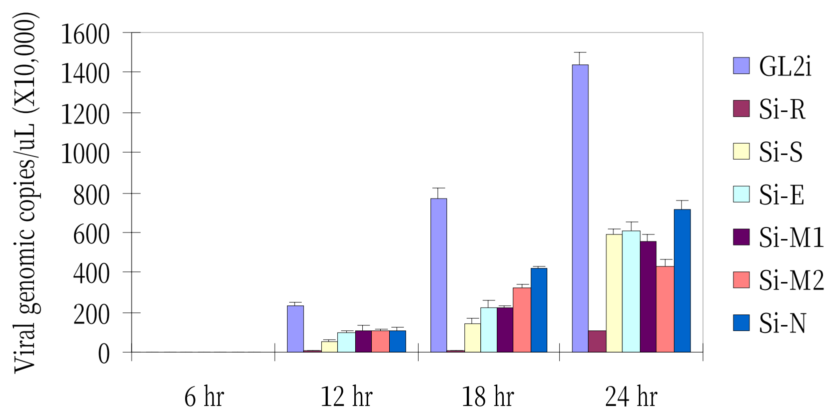

Figure 4. The kinetics of viral genomic RNA. FRhk-4 cells were transfected with siRNAs and infected with SCoV. At 1 hour post-infection the medium containing viruses was removed. The cells were then washed twice with PBS containing 5mM EDTA, and cultured in MEM medium containing 1% FBS. Total RNA was isolated, and viral genomic copies were quantified by real-time RT-PCR.

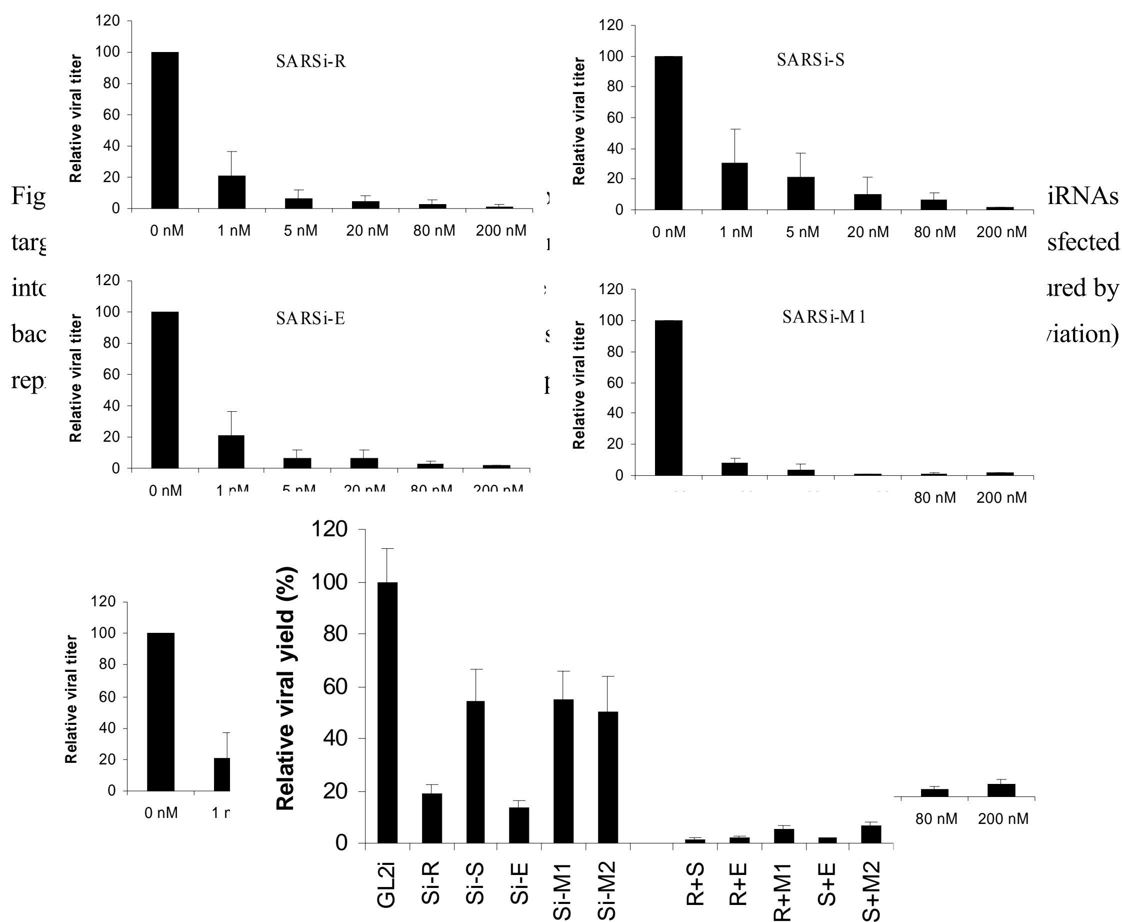

Figure 6. The effects of combined siRNAs. A single siRNA (10 nM), two combined siRNAs (5 nM each siRNA) were transfected into FRhk-4 cells. At 6 hours post-transfection the medium was removed and the cells were infected with SCoV suspended in DMEM for one hour. Then the medium was replaced with DMEM with 1% of FBS. At 24 hours post-infection the viral titers in the conditioned medium were determined by virus infectivity assay. The viral titer of GL2i samples was defined as 100. The values (mean+/-sd) represent the average from three independent experiments.