In Silico and In Vitro Development of novel small interfering RNAs (siRNAs) to inhibit SARS-CoV-2

Noha Samir Taibe  
Cairo University

Sara H. Mahmoud  
National Research Centre

Maimona A. Kord  
Cairo University

Mohamed Ahmed Badawy  
Cairo University

Mahmoud Shehata  
National Research Centre

Mahmoud Elhefnawi  
mahef@aucegypt.edu  
National Research Centre

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Abstract

One of the deadliest pandemics of the 21st century is being driven by SARS-CoV-2, a significant betacoronavirus, causing severe to moderate respiratory tract infections and represents a major public health threat than other human coronaviruses like severe acute respiratory syndrome (SARS) CoV and Middle East respiratory syndrome (MERS), which has been ravaging the world's health, social life, and the economy. In response to the sixth wave of SARS-CoV-2, we aim to develop novel innovative viral replication inhibitor therapeutics.

We achieved highly specific siRNAs by optimizing RNAi efficacy and reducing potential side effects and considering various factors such as target RNA variations, thermodynamics, accessibility of the siRNA, and off-target effects. Out of 258 siRNAs targeting conserved regions, four siRNAs (siRNA1, siRNA2, siRNA3, siRNA4) were chosen based on their predicted potency and high specificity that target critical highly conserved areas (NSP8, NSP12, and NSP14) in the viral genomes of SARS, MERS, and SARS-CoV2 with no predicted human genome off-targets. We test the effectiveness of the four siRNAs on SARS-CoV2 strain hCoV-19/Egypt/NRC-03/2020. In VeroE6 cells, the selected siRNAs at a concentration 100nM had no cellular toxicity. siRNA2 significantly reduced viral replication with a knockdown percentage of 98% after 24 hr post-infection. In addition, siRNA4 had a statistical significance and knockdown percentage, in S gene and ORF1b gene, of 94% in viral replication. SiRNA2 and siRNA4 could be considered as potential siRNA therapy for SARS-CoV-2 infection.

Introduction

In the last 30 years, three new Beta-coronaviruses, namely Severe Acute Respiratory Syndrome (SARS)-CoV, Middle East Respiratory Syndrome (MERS)-CoV, and SARS-CoV2, have emerged and caused significant outbreaks in humans with high case-fatality rates[1, 2]. SARS-CoV2, the cause of COVID-19 (Corona Virus Infectious Disease – 2019), is the most recent addition to human pathogenic coronaviruses (HCoVs)[3]. Coronaviruses (CoVs) are a group of zoonotic viruses that possess the largest and most complex genome (26–32 kb) among positive-stranded RNA (+ RNA) viruses and are enveloped and non-segmented[4]. During discontinuous negative-strand RNA synthesis, the leader and 'body' segments of the sg RNAs are joined, yielding a subgenome-length template for each of the sg mRNAs as shown in Fig. 1[5].

Coronaviruses are positive ssRNA viruses that use ORF1a and ORF1b replicases, making RNA interference (RNAi) a potentially effective therapeutic approach for controlling the virus by targeting viral mRNA at specific stages in human cells[6]. mRNA-based vaccination techniques helped pave the path for a new age of RNA therapies during the current SARS-CoV-2 pandemic. Small interfering RNA-based methods relying on RNAi may supplement clinical COVID-19 care. Targeting both host and protein factors, the RNA interference strategy will largely work by limiting the synthesis of the proteins necessary for viral replication, obstructing viral cellular entry and trafficking[7]. The function of synthetic siRNAs, which have a length of 21–23 bp, is to block post-transcriptional gene expression. Each siRNA is targeted towards a particular gene, unlike miRNAs, thus it can only reduce the expression of that gene[8]. The RNAi approach can be based either on the introduction into cells of lengthy double-stranded RNAs which are subsequently cut into short dsRNAs of around twenty-one base pairs by RNase III (Dicer) in the cytoplasm to form siRNA, or on the direct delivery of siRNA. Later, siRNAs reach the RISC where they become single-stranded RNA (ssRNA). Cellular exonucleases may enter and break the target mRNA if the RISC and siRNA complex could locate the precise target spot on the mRNA [9]as shown in Fig. 1.

Computational methods, Machine Learning[10, 11], are used to build well-targeted and precise siRNAs after first retrieving the genomes of the SARS-CoV-2 virus and its variants. Multiple sequence alignment is used to identify genomic regions that are conserved across different variants[7]. These conserved areas between variants are typically thought of as potential siRNA target locations.

The design of siRNAs is then carried out utilising web servers, incorporating the outcomes of different algorithms according to Ui-Tei[12], Amarzguioui[13], and Reynolds[14] criteria.

For SARS-CoV-2, siRNA prediction, synthesis, and design have all been pioneered by numerous in silico research. Pandey and Verma, 2021[15] performed a study using in silico methods to combat the virus' leader sequence. Another team created siRNA sequences directed at the SARS-CoV2 RNA-dependent RNA polymerase (RdRp) gene and used docking and molecular dynamics modelling to assess how well each siRNA sequence bound to the RdRp gene segment[16].

In this study, we developed four siRNAs with high specificity targeting non-structural proteins (NSP8, NSP12, and NSP14) of human pathogenic coronaviruses, including SARS-CoV, MERS-CoV, and SARS-CoV2. We carefully considered various factors, such as RNA variations, thermodynamics, accessibility, and off-target effects, to enhance RNAi efficiency and minimize potential side effects. However, due to the urgent need to combat the pandemic caused by SARS-CoV2, we tested the siRNAs specifically on this virus.

Materials and Methods

In-silico design of siRNAs:

In this research, we built our in-silico design of siRNAs on the methodologies, shown in Fig. 2, as presented previously in[17] [18] and[19]. The thermodynamic properties of our in silico-designed siRNAs and the characteristics of the siRNAs used in this study are presented in Table 1.
Table 1
Features of siRNAs validated.

<table>
<thead>
<tr>
<th>Name</th>
<th>siRNA sequence(sense)*1</th>
<th>Target*2</th>
<th>Position *3</th>
<th>Thermodynamic properties *4</th>
<th>Target accessibility *5</th>
</tr>
</thead>
</table>

*1 siRNA sequence targeting conserved regions.
*2 conserved regions.
*3 Position on the reference sequence.
*4 Whole ΔG.
*5 total free energy of binding.

Sequence collection:
We focused on the most prevalent three coronavirus species that are categorized as human pathogenic coronaviruses which are Severe Acute Respiratory Syndrome (SARS)-CoV, Middle East Respiratory Syndrome (MERS)-CoV, and SARS-CoV2 the cause of COVID-19. On 5/7/2020, Viral whole-genome sequences were obtained from NCBI’s virus database [https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/].

Multiple sequence alignment and identification of conserved region:
About 12,649 sequences (5,976 from SARS-CoV2, 6,144 from SARS, and 529 from MERS) were used in a multiple sequence alignment process that was carried out using MAFFT version 7 [20]. The alignment output file was analyzed using Jalview [21] to find the most conserved region using a percentage identity threshold of 86.4%. We used BLAST NCBI [https://blast.ncbi.nlm.nih.gov/] to identify the resulting conserved regions through the genome sequence of SARS-COV2 (NC_045512) as shown in Table 2.

Table 2
The final conserved regions and the name of the equivalent protein.

<table>
<thead>
<tr>
<th>NO.</th>
<th>Conserved regions</th>
<th>Blast region</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ATGGCTGATCAAGCTATGAC</td>
<td>NSP8 in ORF1ab</td>
</tr>
<tr>
<td>2</td>
<td>CTGAGGACAAGAGGAGAAGGTA</td>
<td>NSP8 in ORF1ab</td>
</tr>
<tr>
<td>3</td>
<td>GTGTCTAGGGTTTTTCTTAAAGGAGG</td>
<td>RNA-dependent RNA polymerase (NSP12)</td>
</tr>
<tr>
<td>4</td>
<td>TTCTTTGCTCAGGATTGTAATGCTGCTAT</td>
<td>RNA-dependent RNA polymerase (NSP12)</td>
</tr>
<tr>
<td>5</td>
<td>GTGTCTTATAGCTATAGCTTACCC</td>
<td>RNA-dependent RNA polymerase (NSP12)</td>
</tr>
<tr>
<td>6</td>
<td>TTTACTGTTTATCGTGTAAC</td>
<td>ORF1ab Helicase (NSP13)</td>
</tr>
<tr>
<td>7</td>
<td>GTCTTTATTCACCTTATAATTCACAGAATGCTGTCG</td>
<td>ORF1ab Helicase (NSP13)</td>
</tr>
<tr>
<td>8</td>
<td>CAATTTAAACACCTCATAACC</td>
<td>ORF1ab 3’:5’ exonuclease (NSP14)</td>
</tr>
<tr>
<td>9</td>
<td>CATGGCTTTTGGATGGACATCTA</td>
<td>ORF1ab 3’:5’ exonuclease (NSP14)</td>
</tr>
<tr>
<td>10</td>
<td>AAATGGCCATGGTACATTTGGCT</td>
<td>gene S prod: surface glycoprotein</td>
</tr>
</tbody>
</table>
siRNA Design, scoring, and selection steps:

All possible siRNAs were designed for those conserved regions using different online software such as i-SCORE Designer[22], OligoWalk [23], siVirus [24], Sfold [25], SCALES [26], siPRED [27]. As a primary filtration, we used the Huesken dataset[28] to get the 90% predicted experimental inhibition to be able to filter the siRNAs that resulted from i-SCORE Designer using a linear regression model by IBM SPSS Statistics. For the rest of the previous software programs, we used a certain accepted threshold by assigning accepted threshold scores for these tools. Then We selected the best siRNAs based on what exceeds all the previous thresholds as shown in Additional file 1.

Thermodynamics and Target Accessibility:

As the siRNA duplex stability is very important, we used RNAfold[29], SiRNA[25], to calculate RNA duplex thermodynamics for the designed siRNA and the difference in 5’ end terminal free energy (ddG) of the sense and anti-sense strands. Then we selected the best siRNAs that have dG values between −35 and −27 kcal/mol[30]. We also used Sfold[25] and RNAfold[29] to know the secondary structure of the siRNA with target regions in the mRNA of SARS-CoV2. To be sure about the target accessibility of the designed siRNAs with their targets, we used IntaRNA2.0[31] and RNAup[29].

Off-target Filtration and Final selection for designed siRNAs:

We used seed region and complete sequence of siRNAs in NCBI BLAST with human mRNA RefSeq set [32] to avoid any near-complete or seed region matches with other vital genes that may cause downregulation of these genes using the parameters provided in the classical Birmingham protocol[18]. After all those stages of filtration, we selected the highest predicted efficacy and specificity of the designed siRNAs to be tested experimentally as shown in Additional file 1.

In vitro methods:

siRNAs preparation:

All four designed siRNAs, shown in Table 1, were custom-made (Dharmacon, Horizon Discovery, UK). A Positive control siRNA (targeting GAPDH)5-GUAUGACAACAGCCUCAAGTT-3(forward) and 5_CUUGAGGCUGUUGUCAUACTT-3 (reverse) and a Negative control siRNA (non-targeting) 5-UUCUCCGAACGUGUCACGUTT-3(forward) and 5-ACGUGACACGUUCGGAGAATT-3[33], were custom-made (Dharmacon, Horizon Discovery, UK). We prepared siRNAs according to the siRNA resuspension protocol (Horizon Discovery, UK) and aliquoted then stored them at -20°C.

virus propagation:

Vero E6 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS (Fetal bovine serum) 1% Penicillin/Streptomycin (pen/strep) antibiotic mixture at 37°C, 5% CO2. hCoV-19/Egypt/NRC-03/2020 (GISAID accession number: EPI_ISL_430819)[34] was grown in Vero E6 cells. All SARS-CoV infection experiments were conducted under biosafety level-3 conditions (BSL-3).

Cytotoxicity (CC50) determination:

The siRNAs were dissolved in 1x siRNA buffer (catalogue ID: B-002000-UB-100, Dharmacon, Horizon Discovery, UK) at a concentration of 10µM to determine the CC50. The siRNA stock solution was further diluted to working solutions (10 dilutions) using DMEM (10 µM − 0.0195 µM). The crystal violet assay was used to assess cytotoxicity in VERO-E6 cells, as previously described[35]. To measure the half-maximal cytotoxic concentration (CC50), the siRNAs were serially diluted (Bi-fold dilutions) in DMEM medium.

Briefly, the cells were seeded in 96-well plates (100 µl/well at a density of 3 ×10^5 cells/ml) and incubated at 37°C in 5% CO2 for 24 hours. After 24 hours, the cells were treated in triplicate with different doses of siRNAs. The supernatant was discarded 72 hours after treatment, and cell monolayers were fixed with 10% formaldehyde for 1 hour at room temperature (RT). The fixed monolayers were properly dried before being stained with 50 µl of 0.1% crystal violet on a bench rocker at room temperature for 20 minutes. After washing and drying the monolayers overnight, the crystal violet dye in each well was dissolved in 200 µl methanol for 20 minutes on a bench rocker at room temperature. The absorbance of the crystal violet solutions was measured at λmax 570 nm as a reference wavelength using a multi-well plate reader. The CC50 value was calculated using nonlinear regression analysis using GraphPad Prism software (version 5.01) by plotting log concentrations of the compound versus normalized response (variable slope). The percentage of cytotoxicity compared to the untreated cells was determined with the following equation.

\[ \text{Cytotoxicity} = \frac{\text{absorbance of cells without treatment} - \text{absorbance of cells with treatment}}{\text{absorbance of cells without treatment}} \times 100 \]

The concentration that displayed 50% cytotoxicity (CC50) was calculated using a plot of percent cytotoxicity vs sample concentration[36].

Virus propagation and titration:
SARS-CoV-2 strain hCoV-19/Egypt/NRC-03/2020 (GISAID accession number: EPI_ISL_430819) was propagated in Vero-E6 cells to generate virus stock in the presence of L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin. Virus stock was propagated in Vero-E6 cells cultured at a multiplicity of infection (MOI) 0.001, then cells were microscopically investigated daily. The virus-infected culture supernatant was clarified by centrifugation at 4000 rpm for 15 min at 4°C twice. The harvested virus was titrated by TCID\textsubscript{50} assay.

**Titration of virus (TCID\textsubscript{50} assay):**

hCoV-19/Egypt/NRC-03/2020 SARS-CoV-2 isolate virus was serially diluted (half log dilution from neat to log-7.5) using a 96-well dilution plate. A total of 230 µl of DMEM with 2% BSA (Bovine Serum Albumin) (infection media) was added to wells (from A to G in column 1 and from A to H in column 5 b) and 300 µl of the virus was added to column 1 row H. 105 µl of the virus was transferred from row H column 1 to row G column 1. This process was repeated until the achievement of log 7.5, and series dilution was repeated with the changing of filter pipette tips each time. 96 wells Vero-E6 confluent cells plate was prepared by washing the cells two times with an infection medium. A volume of 35µl of each viral dilution was applied to Vero-E6 cells in 96 well micro-titer dilution plates in quadruplicate to cell plates and cell controls were kept in the same plate. Plates were incubated for 1 hr at 37°C with 5% CO\textsubscript{2} incubator then virus dilutions were removed, and the plates were washed with 1X PBS. 150µl of infection medium was added to the Vero cell plate. The VeroE6 cell plate was incubated at 37 C in 5% CO\textsubscript{2} incubator for 3 days. Finally, the cytopathic effect (CPE) was examined and observed and TCID\textsubscript{50}/35µl was calculated according to (Reed and Muench, 1938).

**Transfection:**

Vero E6 cells (10\textsuperscript{5}) infected with the SARS-CoV-2 virus was seeded in 12 well plates, at a multiplicity of infection (MOI) 0.01, then cells were microscopically investigated daily until reached 70% confluence. Cell cultures were transfected with 100 nM (according to Basic siRNA Resuspension Protocol of Dharmacon, Horizon Discovery, UK) of siRNA1, siRNA2, siRNA3, siRNA4, positive siRNA, negative siRNA according to Lipofectamine 3000 protocol. Transfected Vero E6 cells with Sars-CoV-2 isolate were used as a control and Vero E6 cells with it as untreated control.

**RNA extraction and quantitative RT-PCR:**

Viral RNA was extracted from eight-cell cultures at the indicated time points using QIAamp Viral-RNA Kit (Qiagen, Germany). cDNA was synthesized from total RNA using High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems. Nanodrop (NANODROP 2000c, ThermoScientific) was used to determine the quantity and quality of cDNAs. Three monoplex real-time RT-PCR assays targeting the ORF1b and S gene regions of SARS-CoV-2 and human GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) gene as a control (reference gene). The primer sequences, present in Table S1, are synthesized by Macrogen. A typical 20µl reaction contained 10µl Maxima SYBER Green qPCR Master Mix(2x), no ROX (ThermoFisher), 0.6µl forward primer(10pmol/µl), 0.6µl reverse primer(10pmol/µl), ROX solution 0.04µl, 1µL Template DNA, 7.76µl water, nuclease-free. RT-PCR reactions were conducted by a thermal cycler (Applied Biosystem 7500 Fast) with the following conditions: 1 cycle of initial denaturation at 95°C for 10 min, 40 cycles of PCR amplification (Denaturing at 95°C for 15 s, Annealing/Extending at 60°C for 60 s). we used these steps:

- Step 1. Normalize to (Reference Gene): \( \Delta C_q = C_q (\text{target gene}) - C_q (\text{reference gene}) \)
- Step 2. Exponential expression transform: \( \Delta C_q \ \text{Expression} = 2^{-\Delta C_q} \)
- Step 3. Average replicates and calculate the standard deviation.
- Step 4. Normalize to treatment control.
- Step 5. % KD = (1 – \( \Delta \Delta C_q \)) \times 100.

as described in [37, 38] to analyze the data to get relative gene expression and percentage of the knockdown.

**Statistical Analysis:**

All data were obtained in triplicate in at least three independent trials. The acquired data were coded, tabulated, and statistically analysed using GraphPad Prism, version 5.01 (GraphPad Software). The data are shown as mean standard deviation (SD). To compare the differences between the groups, statistical analysis was carried out using two-way ANOVA followed by the Tukey-Kramer multiple comparisons test. A duplicate of each sample was used in the qRT-PCR experiment to quantify relative gene expression using unpaired t-test in GraphPad Prism, version 8.01. (GraphPad Software). The difference in significance is shown by the symbols *p < 0.0332, **p < 0.0021, ***p < 0.0002, ****p < 0.0001; ns( not significant) p < 0.1234.

**Results**

**In-silico design and selection of siRNAs targeting coronaviruses**
After performing whole-genome alignment to 12,649 sequences of the most common human pathogenic coronaviruses which are (SARS)-CoV, (MERS)-CoV, and SARS-CoV2 using MAFFT version 7[20]. 10 conserved regions were identified as RNAi target regions after setting up a percentage identity threshold of 86.4% using Jalview2.11[21], we chose this percentage to have conserved regions consisting of at least 20 nucleotide. The representative alignment (Additional file 2: Fig. S1) showcasing the alignments of the 10 conserved regions identified in this analysis against the reference sequence of SARS (NC_004718.3), SARS-CoV-2(NC_045512.2), and MERS (NC_019843.3) using Clustal Omega. Table 2 contains where the conserved regions can be found on the SARS-CoV-2 genome reference sequence NC_045512.2. About 258 siRNAs were designed by using multi-online software as i-Score Designer[22], OligoWalk [23], siVirus [24], Sfold [25], SCALES [26], siPRED [27], to target the resulting conserved regions. After primary filtration of the predicted experimental inhibition cutoff, we got 56 siRNAs. We calculated their thermodynamic features as Whole ΔG for RNA duplex and differential stability of 5 and 3 end. We filtered them according to [30] as the most preferable ΔG of efficient siRNA ranges between ~35 and ~27 kcal/mol. From this step, we got only 20 siRNAs. So, we checked the secondary structure of the siRNA with target regions in mRNA of SARS-CoV2 through Sfold and RNAfold[29] and also, their target accessibility by using IntaRNA2.0[31] and RNAup [29]. Only 10 siRNAs resulted from the second filtration step. The last stage of filtration was off-target selection, we used BLAST NCBI to reject any siRNA that has another complete and partial match with the human mRNA RefSeq set[7]. After performing all those stages of filtration, we got only 4 siRNAs that can describe them as the most predicted specific and efficient designed siRNAs. The features of the final selected siRNAs that are used in this study, based on the previous steps, are presented in Table 1.

**siRNA cytotoxicity (CC50) on Vero E6 cells**

The cytotoxicity of six siRNAs (siRNA1, siRNA2, siRNA3, siRNA4, positive siRNA, negative siRNA) was tested in Vero E6 cells using a crystal violet assay, and the cytotoxic effects of the designated siRNA were shown to be concentration dependent. All generated siRNAs had virtually comparable results, exhibiting good viability at 100 nM. Figure 3 shows that none of the tested siRNAs were particularly cytotoxic to Vero E6 cells, and the CC50 for all siRNAs was greater than 10 µM.

**Small interfering RNAs (siRNAs) reduce SARS-CoV-2 replication**

At 0,12,24,36,48 hours post-infection, the supernatant of siRNA1, siRNA2, siRNA3, siRNA4, and negative siRNA was collected to measure the virus titer. All siRNAs reduced viral gene expression with varying efficacy, and all data were standardized to the Negative siRNA (scrambled siRNA). At 12 hours, the effect/differences of four siRNAs were highly significant with a P-value of 0.05, siRNA 2 inhibits viral growth the most (2.3 log 10 TCID50) compared to Negative siRNA (4.5 log 10 TCID50). Figure 4 indicates that siRNA 2 continues to inhibit viral replication after 24, 36, and 48 hours with 2.5, 1.5, and 4.5 log 10 TCID50, respectively. When compared to siRNA 1 and siRNA 4, siRNA 3 inhibits viral growth by 3.34 and 3.5 log 10 TCID50 at 24 and 36 hours, respectively. At 48 hours, as shown in Fig. 4, siRNA4 has a significant P value of 0.05 with 7.3 log 10 TCID50 compared to 9.5 log 10 TCID50 of Negative siRNA (scrambled siRNA).

**qRT-PCR quantifies siRNAs antiviral inhibition activity:**

After 12 h.p.i, siRNA2 reduces S gene expression with a great significance P value < 0.0021, while siRNA1 and siRNA4 reduce S gene expression with P value < 0.0332 (Additional file 2: Fig. S2). All siRNAs exhibit a significant P value in the reduction of S gene mRNA at 24 hours post-infection, as shown in Fig. 5. siRNA2 still shows the highest P value < 0.002 after 36h.p.i. (Additional file 2: Fig. S3). After 48h.p.i, the effect of siRNAs in gene silencing decreases, (Additional file 2: Fig. S4), but the siRNA2 still shows the reduction in S gene expression with P value < 0.0332 and 59% knockdown.

siRNA2 and siRNA4 show a great significant knockdown of ORF1b gene after 12h.p.i with P value < 0.0002 (Additional file 2: Fig. S5). siRNA2 shows the great significance in reduction ORF1b expression with P value < 0.001 after 24 and 36h.p.i, While siRNA4 shows P value < 0.0002 after 24h.p.i and P value < 0.0021 after 36h.p.as shown in Fig. 6 and (Additional file 2: Fig. S6) respectively. siRNA3 shows a significant P value < 0.0021 after 24 and 12h.p.i as shown in Fig. 6 and (Additional file 2: Fig. S5) respectively. Even though siRNAs’ impact decreased after 48 hours post-infection, siRNA2 and siRNA4 still show a significant reduction in expression of ORF1b with P value < 0.0332 (Additional file 2: Fig. S7).

**Discussion**

To date, approximately seven million people have died as a result of the COVID-19 pandemic, which was caused by a positive-sense RNA virus known as SARS-CoV2. Traditional vaccine development takes 6–8 months; during this time, the virus undergoes several mutations in the candidate protein chosen for vaccine development[39]. By the time the protein-based vaccine is available, the virus will have undergone several mutations, and antibodies against the viral sequence may no longer be effective in limiting newly mutated viruses[40]. Genetic variance analyses of the entire genome in 48,635 SARS-CoV2 samples, compared to the reference genome (Wuhan genome) NC_045512.2, revealed a reasonable average of 7.23 mutations per sample[41]. The SARS-CoV2 genome’s proclivity for adaptive mutations may have made it extremely pathogenic, complicating drug and vaccine development[42]. SARS-CoV2 genetic variations, even within the same country, pose a challenge to finding a universally applicable therapeutic agent[43, 44]. Challenges of treatment necessitate a new dimension, especially when an effective antiviral agent is required. Several
drugs used to treat SARS-CoV and MERS-CoV were discovered to be ineffective against SARS-CoV2[45]. Due to the lack of SARS-CoV-2 specific drugs, we are looking for an effective and specific therapeutic approach. RNAi(RNA interference) is a gene-silencing mechanism that can be activated by siRNA[46] and has the potential to prevent pathogenic viral replication and infection in animal cells[47]. HCV[48], Influenza[49] and HIV were restricted using siRNA-silencing technology[50]. According to recent research, siRNAs can suppress gene expression, which prevents SARS and MERS viral replication in cultured cells[51–54].

These investigations concentrated on the specific SARS-CoV genes where there would be a high likelihood of single nucleotide alterations, decreasing the efficiency of siRNA targeting[51]. This effort may pave the way for precision/personalized medicine to treat SARS-CoV2 patients. This motivates us to consider specific siRNA-based therapeutics based on conserved and diverse potential targets in SARS-CoV2 genome reference sequences[55].RNAi technology has the potential to suppress SARS-CoV2 viral replication by generating sequences based on the status of viral mutations in actual time[56]. SARS-CoV-2 genome contains 14 Open Reading Frames (ORFs) and 27 proteins[52]. ORF1a and ORF1b have highly preserved sequences in the annotated genomes of SARS-CoV2 and earlier beta coronaviruses like SARS and MERS[57]. The siRNAs have the ability to silence the targeted genes while also inhibiting virus replication. Previously reported SARS virus studies were similar[51].

In this research, we select Four siRNAs that are the most predicted specific and efficient designed siRNAs by using bioinformatics approach. Those siRNAs target specific conserved regions in the SARS-CoV2 genome. These regions are responsible for the encoding of non-structural proteins (NSPs) 8,12 and 14. Many important enzymes for RNA processing and viral replication are encoded by these NSPs, including the RNA-dependent RNA polymerase (RdRp) and N7-guanine methyltransferase(MTase). NSP8 is critical in extending the template RNA-binding surface as a cofactor with NSP7 to bind to NSP12 as a viral polymerase complex. NSP8 functions as an innate immune suppressor, promoting viral replication and transcription[58]. NSP12, also known as the viral RdRp, possesses the catalytic activity required for viral replication. NSP12 has little activity on its own and needs the cofactors NSP7 and NSP8 to synthesize RNA. NSP7 and NSP8 function as primases, stabilizing the RNA-binding region of NSP12 for SARS-CoV2 genome replication. SARS-CoV and SARS-CoV-2 NSP12 are 96–98 percent identical, implying that their structure and function are likely to be identical[59]. NSP14 is a highly conserved NSP known for its 3' to 5' ExoN activity as well as guanine -N7-MTase activity, the latter of which mediates RNA capping in collaboration with NSP12, NSP13, and NSP16. NSP14 is an S-adenosylmethionine (SAM)-dependent MTase that uses SAM as a methyl donor, which is required for viral replication[60].

In our in vitro studies, before inducing virus infection and qPCR, the cellular toxicity of each designed siRNA was evaluated in the Vero E6 cell line. The results showed that the tested siRNAs had no cytotoxicity in the tested cell lines. In Vero cells, after transfection, siRNA 2 shows a highly significant and strong inhibitory effect of 98% on viral replication compared with siRNA negative (scrambled). The reduction in viral replication due to the low production in NSP8 (Primase) that consider a cofactor for NSP12. NSP12 and NSP8 play critical roles in the formation of the entire RNA polymerase replicative machinery[61]. After 36 h post-infection, siRNA3 shows a high reduction in viral growth with 73% due to it is effect in silencing NSP14, essential for viral replication and transcription. CoV NSP14 is required for viral replication and transcription, in the case of SARS-CoV-2, The ExoN domain of NSP14 functions as a proof-reader, preventing lethal mutagenesis, whereas the C-terminal domain is a methyltransferase for mRNA capping. In addition to high-fidelity replication, ExoN is thought to be important in RNA synthesis, resistance to antiviral nucleoside analogues, fitness, immune antagonism, and virulence. It has also been linked to increased recombination, which is necessary for virus evolution[61]. During the 2020 pandemic, NSP14 had a very low rate of mutational variation. Only M501 and N129 showed mutational rates greater than 0.01[62]. siRNA4 also shows a 94% reduction in viral growth at 12,24,36,48 h post-infection. Reflect it is effective in silencing NSP12, an RNA-dependent RNA polymerase (RdRp), forms a viral replication complex with NSP7, NSP8, and other essential components of the RNA synthesis machinery[58]. qRT-PCR testing is a widely used and highly specific messenger RNA detection and quantification technique that can detect SARS-CoV-2 in biological specimens. The amount of viral RNA in the sample correlates inversely with the cycle threshold (Ct) value. We used structural gene as the spike (S)gene and species-specific accessory genes that aid in viral replication, such as the open reading frame 1b (ORF1b) gene, according to Centers for Disease Control and Prevention (CDC) and World Health Organization (WHO) guidelines[63–65]. siRNA2 significantly decreases the viral replication, knocking down NSP 8 gene by 98%, through disruption of the viral NSP7-NSP8-NSP12 holo-RdRp RTC(replication–transcription complex)[66]. siRNA3 shows 73% knockdown in the NSP 14 gene, via silencing of the NSP 14 gene, this leads to a formation disturbance in the RNA proofreading complex that will help the host proteases in degradation of the viral genome and activate the immune system to fight the viral infection[67]. siRNA4 reduce transcription of NSP12 gene with 94%. NSP12 is an essential enzyme in the virus life cycle, the coronavirus RTC relies on NSP12, not only for viral genome replication but also for sgRNA transcription[66]. According to our findings, siRNA therapy can significantly lower viral load when compared to other research like Friedrich et al. (2022)[68] and Niktab et al. (2021)[69]. Our future work, we will use modified oligonucleotides[70] for therapeutic delivery of the siRNAs in in-vivo studies.

Conclusion

There is no vaccine or drug that is 100% effective against COVID-19[71]. The FDA recently approved several siRNA-based therapeutics after many obstacles and challenges were overcome. Based on our computational approach, as well as the experimental evaluation of selected siRNAs for cytotoxicity and qPCR, We concluded that siRNA 2 and siRNA4 could be considered potent new therapies for SARS-CoV-2 that need to be developed further.
Declarations

Data Availability Statement:

The data that support the findings of this study are available upon reasonable request to the corresponding author.

Acknowledgments:

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Authors’ contributions:

N.S: conceived the work, performed in-silico design of siRNAs and RT-PCR experiment, wrote the manuscript, drew the Figures, and share in putting the main idea.

S.H.M: performed the experiments that deal with SARS-CoV2 virus and revised the manuscript.

M.E: put the main idea, conceived the work, revised the manuscript, and supervised the work.

M.K, M.B, M.S: supervised the work.

All authors read and approved the final manuscript.

Declaration of Interests:

N.S, S.H.M, M.E submitted US provisional patent (Application number:63444249)

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Figure 1

Schematic representation of siRNA’s gene silencing mechanism.

SARS-CoV-2 binds to the surface receptor angiotensin-converting enzyme 2 (ACE-2) on the host cell and releases its RNA genome in the cytoplasm via endocytosis or direct membrane fusion. After siRNA transfection using lipofectamine3000, The active RISC is then guided to the target mRNA by the guide strand of siRNA. The complete complementary binding of the guide strand of siRNA to the target mRNA results in mRNA cleavage. Created with BioRender.
Figure 2

Flowchart of in-silico siRNA design steps and methodology.

Stage 1 and 2: viral genome sequences were selected and aligned. Stage 3: 258 siRNAs were designed by various online software. 56 siRNAs resulted from scored according to the Huesken dataset and 90% experimental threshold inhibition scores were determined. Stage 4: 20 siRNAs resulted after performing thermodynamics features using RNAfold and Sima. Also, after performing target accessibility using IntaRNA and RNAup we got 10 siRNAs. Stage 5: Final filtration, we used NCBI BLAST to reject any siRNA that has a complete match with the human mRNA RefSeq set. 4 siRNAs were selected for the in-vitro experiments. Created with BioRender.
Figure 3

Cytotoxicity (CC50) of siRNAs on Vero E cells

Using a crystal violet assay, the cytotoxicity of six siRNAs (siRNA1, siRNA2, siRNA3, siRNA4, positive siRNA, negative siRNA) was tested in Vero E6 cells, and the cytotoxic effects of the designated siRNA were found to be concentration dependent. All siRNAs produced nearly identical results, with good viability at 100 nM. None of the tested siRNAs were particularly cytotoxic to Vero E6 cells, and the CC50 for all siRNAs was more excellent than 10 M. The data is presented as the mean ± S.D of 3 independent replicates. GraphPad Prism (GraphPad Software, version 5.01) was used to code, tabulate, and statistically analyze the collected data.
Figure 4

Replication Efficiency of infected VeroE6 cells at 0, 12, 24, 36, and 48 hr post-infection using TCID50 assay at MOI 0.01

Vero E6 cells were transfected with SARS-CoV-2 and the six siRNAs at 100nM concentration.

All data were collected in triplicate at 0, 12, 24, 36, and 48 hours post infection. GraphPad Prism Software, version 5.01, was used to code, tabulate, and statistically analyze the collected data. The data is presented as the mean ± standard deviation (SD).
Figure 5: Knockdown effect of siRNAs transfection on S gene in infected VeroE6 cells

After 24h.p.i, all siRNAs highly reduced viral replication B: siRNA2 shows high significance in reduction of mRNA of SARS-CoV-2 with P value <0.0002 compared to A: siRNA1, C: siRNA3, D: siRNA4. E: the knockdown percentage of all siRNAs compared with scramble siRNA. All results were normalized against scramble siRNA and quantitative examination (n = 2 in each group). By GraphPad Prism, version 8, means ± SEM is used to represent the values. significance was determined using unpaired t-test. The significant difference is shown by the symbols *p<0.0332, **p< 0.0021, ***p< 0.0002, ****p <0.0001; ns (not significant) p< 0.1234.

Figure 5

Knockdown effect of siRNAs transfection on S gene in infected VeroE6 cells

After 24h.p.i, all siRNAs highly reduced viral replication B: siRNA2 shows high significance in reduction of mRNA of SARS-CoV-2 with P value <0.0002 compared to A: siRNA1, C: siRNA3, D: siRNA4. E: the knockdown percentage of all siRNAs compared with scramble siRNA. All results were normalized against scramble siRNA and quantitative examination (n = 2 in each group). By GraphPad Prism, version 8, means ± SEM is used to represent the values. significance was determined using unpaired t-test. The significant difference is shown by the symbols *p<0.0332, **p< 0.0021, ***p< 0.0002, ****p <0.0001; ns (not significant) p< 0.1234.
Figure 6: Knockdown effect of siRNAs transfection on ORF1b gene in infected VeroE6 cells

After 24h.p.i, all siRNAs highly reduced viral replication B: siRNA2 shows high significance in reduction of mRNA of SARS-CoV-2 with P value <0.0001 compared to A: siRNA1, C: siRNA3, D: siRNA4. E: the knockdown percentage of all siRNAs compared with scramble siRNA. All results were normalized against scramble siRNA and quantitative examination (n = 2 in each group). By GraphPad Prism, version 8, means ± SEM is used to represent the values. significance was determined using unpaired t-test. The significant difference is shown by the symbols *p<0.0332, **p< 0.0021, ***p< 0.0002, ****p <0.0001; ns (not significant) p< 0.1234.

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