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Article

Anticipating the next chess move: Blocking SARS-CoV-2 replication and simultaneously disarming viral escape mechanisms

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Abstract: The COVID-19 pandemic initiated a race to determine the best measures to control the disease and to save as many people as possible. Efforts to implement social distancing, the use of masks, and massive vaccination programs turned out to be essential in reducing the devastating effects of the pandemic. Nevertheless, the high mutation rates of SARS-CoV-2 challenge the vaccination strategy and maintain the threat of new outbreaks due to the risk of infection surges and even lethal variations able to resist the effects of vaccines and upset the balance. Most of the new therapies tested against SARS-CoV-2 came from already available formulations developed to treat other diseases, so they were not specifically developed for SARS-CoV-2. In parallel, the knowledge produced regarding the molecular mechanisms involved in this disease was vast due to massive efforts worldwide. Taking advantage of such a vast molecular understanding of virus genomes and disease mechanisms, a targeted molecular therapy based on siRNA specifically developed to reach exclusive SARS-CoV-2 genomic sequences was tested in a non-transformed human cell model. Since coronavirus can escape from siRNA by producing siRNA inhibitors, a complex strategy to simultaneously strike both the viral infectious mechanism and the capability of evading siRNA therapy was developed. The combined administration of the chosen produced siRNA proved to be highly effective in successfully reducing viral load and keeping virus replication under control, even after many days of treatment, unlike the combinations of siRNAs lacking this anti-anti-siRNA capability. Additionally, the developed therapy did not harm the normal cells, which was demonstrated because, instead of testing the siRNA in nonhuman cells or in transformed human cells, a non-transformed human thyroid cell was specifically chosen for the experiment. The proposed siRNA combination deeply reduced the viral load throughout the experiment and allowed cellular recovery, thus representing a potential innovation, to be considered as an additional weapon for therapy of COVID-19 and even other infectious diseases.

Keywords: COVID-19; SARS-CoV-2; siRNA; Treatment.

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the etiologic agent of COVID-19 (Coronavirus Disease, 2019), the cause of the present pandemic. The clinical manifestations range from asymptomatic to acute respiratory distress syndrome (ARDS), severe pneumonia [1] and even patient death, since there are no specific treatment options to treat this disease.

Additionally, the persistence of some of the symptoms of the disease after the acute phase of SARS-CoV-2 infection, reducing their quality of life, has been reported.

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It was observed that within 6 months of the onset of the first symptoms, approximately 76% of patients reported the emergence and/or persistence of at least one symptom of the disease during the follow-up period. The most frequent resilient symptoms were muscle weakness or fatigue, sleeping difficulties, anxiety, depression, and impaired pulmonary diffusion [2].

Global efforts to control COVID-19 resulted in a race for potentially efficient therapies against SARS-CoV-2; nevertheless, these initiatives did not result in great advances, i.e., clinical trials were deeply disappointing, and the development of new therapeutic approaches with strong results has yet to occur [3].

The speed of vaccine development conflicts with the speed of virus mutations that enable the permanency of very high indices of infections and deaths and decrease vaccine efficacy over time, making disease control a major challenge [4].

Although the combination of population vaccination and protective measures such as facemasks, hygienic protocols, and social distancing greatly contributed to COVID-19 control, the world is still at risk of losing this fragile equilibrium due to the threat of new epidemics [5].

Most of the initiatives to find a putative treatment against SARS-CoV-2 are based on testing many molecules and drugs used for other diseases, aiming to discover a secondary benefit against COVID-19; however, unfortunately, no drug was found to present or support an efficient and specific treatment [6].

Although vaccines and protective measures have saved millions of lives, efficient and real control of COVID-19 was not reached Intriguingly, each great scientific advance for controlling the pandemic was countered by the emergence of more aggressive and/or infectious variants of SARS-CoV-2 that changed the game in favor of virus success [7].

Small interfering RNAs (siRNAs) are nucleotide sequences able to specifically inhibit complementary RNA sequences in a highly effective way, thus preventing strikes on incorrect targets and efficiently blocking the target sequences. Therapeutic application of siRNAs is currently in clinical practice due to their high efficiency and safety [8].

Since SARS-CoV-2 is an RNA virus and considering previous promising initiatives using siRNAs to block the replication of coronavirus, targeted antiviral therapy using siRNAs should be attempted, in addition to the current strategy of providing vaccination and other protection measures to control COVID-19 [9,10].

Aiming to selectively impair viral replication and simultaneously anticipate viral reactions, including an siRNA escape mechanism, as previously described, i.e., preventing a SARS-CoV-2 escape strategy that could reduce the sustained therapeutic response, a combined targeted therapy using siRNAs directed to both combat the virus and its siRNA escape mechanism was developed and tested in a cellular model of no transformed human cells. The proposed strategy has proven to be safe and extremely efficient in consistently reducing viral load while reestablishing cellular viability.

2. Materials and Methods

2.1. Virus isolate

The SARS-CoV-2 strain used in the experiments was taken from the sample bank belonging to the Instituto Evandro Chagas. To confirm the case of SARS-CoV-2 infection, the previously described RT–qPCR[11] was performed using the commercial kit GoTaq Probe 1-Step RT–qPCR (Promega, USA).

For the isolation of positive samples, the cell culture was performed using VERO E6 cells as described elsewhere [12]. After cultivation into Vero cells and when 75% of cells showed cytopathic effects (CPEs), the culture was harvested and centrifuged at 1000 g/min for 5 min, and the supernatants were stored in aliquots at -80 °Cuntil use.

2.2. The nontransformed in vitro model

Nontransformed human thyroid cells (IMR-90) were cultured in DMEM as described elsewhere [13]. An MOI of 0.5 of the SARS-CoV-2 strain BIO01/2020 was used to infect the

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cells. The culture was maintained for 120 hours post-infection (hpi), thus proving to be a good model for the siRNA experiment. This same infection protocol was used for all experiments.

2.3. Viral Load

The method described by [14] was used to quantify the viral load. Briefly, 24-well plates seeded with VERO E6 cells at a concentration of 1×10^4 cells/well were used. After 24 h and 80-90% confluence, dilutions from 10^{-1} to 10^{-10} in DMEM containing 2.5% fetal bovine serum (FBS) of the virus were transferred in triplicate (100 µL/well) to seeded plates. After 1 hour of adsorption at 37 °C in a 5% CO_2 atmosphere, the wells were completed with an overlay of carboxymethylcellulose (CMC) with DMEM, 2% FBS and 1% penicillin–streptomycin mix, and the plates were incubated at 37 °C in a 5% CO_2 incubator and stained with methylene blue dissolved in sodium acetate-acetic acid. Plates were stained after 96 hpi. Finally, the viral load in PFU/ml of each sample was determined.

2.4. Treatment with siRNA

A total of four siRNAs (siRNAs I, II, III and IV) targeting different sequences in the 1a/1ab ORF regions were used both individually and together. A negative control (SC2_NC) was also applied, as recommended by the producers, ensuring that the siRNA system was working properly. These siRNAs were mixed with the RNAiMax Lipofectamine delivery vehicle (Thermo Fisher Scientific, USA) at a concentration of 500 ng/well of Lipofectamine for every 5 pmol/well of siRNA, and the same concentrations were used for the SARS-CoV-2 uninfected controls.

The Silencer Select GAPDH siRNA (Ambion, USA) and Silencer Select Negative Control (Ambion) systems were also applied as quality controls.

In addition, an extra siRNA, the anti-escape siRNA (siRNA-V), was combined with siRNA-I plus siRNA-III for the additional experiments, keeping the same described protocols except regarding the number of treatments, since instead of giving daily doses, the combinations of both siRNA-I plus siRNA-III and siRNA-I plus siRNA-III plus siRNA-V were applied exclusively 24 hpi as unique doses.

2.5. Experimental design

All experiments were performed at least three times to ensure data reliability. An interval of 24 hours between siRNA treatments for the removal of both the cell supernatant for viral quantification and the cells for the proposed analysis was adopted (Supplement 1).

2.6. Analysis of Cytotoxicity, Cell Viability and Cytopathic Effect

After infecting the cells with the SARS-CoV-2 strain, the analysis of cytotoxicity, caspase 3 and caspase 7 signaling and cell viability was performed using the ApoTox-Glo Triplex Assay; the induced cytopathic effects (CPEs) were quantified by a Viral ToxGlo Assay (Promega). Mitochondrial activity was also analyzed using the Mitochondrial ToxGlo Assay kit (Promega).

2.7. IMR-90 cell infection, viral loads and cell viability

An MOI of 0.5 was used to infect IMR-90 cells to maintain both SARS-CoV-2 replication and cell viability over 120 hpi, allowing culture observation and viral load estimation (Appendix A).

Since the cells needed to remain viable for at least five days after infection to allow the *in vitro* treatment experiment, the measurements were quantified over five days, and the results are shown in Appendix B (C), confirming that the model works properly. Even with an evident decay of cell number occurring at 48 hpi, most cells remained viable until 120 hpi.

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2.8. The cytopathic effect demonstration

The SARS-CoV-2 culture showed evident morphologic CPEs (Supplement 1-A). In addition, ATP production was also quantified using the Glo-max platform, since the level of ATP production is directly related to CPEs (Appendix C-B). The expression of caspases 3 and 7 (Supplement 3-B) and mitochondrial viability (Appendix C-A) were analyzed, and a deterioration of cellular homeostasis was demonstrated.

Finally, the relationship between viral load, cell viability and CPEs, as well as between viral load and caspase expression (Appendix C), demonstrated a deterioration of cellular homeostasis related to the increase in viral load.

Since the established model proved to be useful for the designed siRNA experiment, the next steps included the evaluation of the siRNA's toxicity to the cells, mainly regarding any off-target effect, to warrant strategy safety (Appendix D).

2.9. Statistical analysis

The R-project program (r-project.org/) and the JAMOVI program v. 1.6 (jamovi.org) were used. ANOVA and Student's t tests were used for group comparisons. A significance value of $p<0.05$ and a 95% confidence interval were adopted.

3. Results

3.1. siRNA treatments

Initially, each siRNA was tested individually to verify the differences in viral load throughout the treatment and the efficacy of each siRNA (Figure 01). In addition to the viral load, cell viability was also verified throughout the experimental treatment. Next, a "cocktail" was created using every four siRNAs together. The siRNA combination increased treatment effectiveness when compared to each individually tested siRNA (Figure 01-E).

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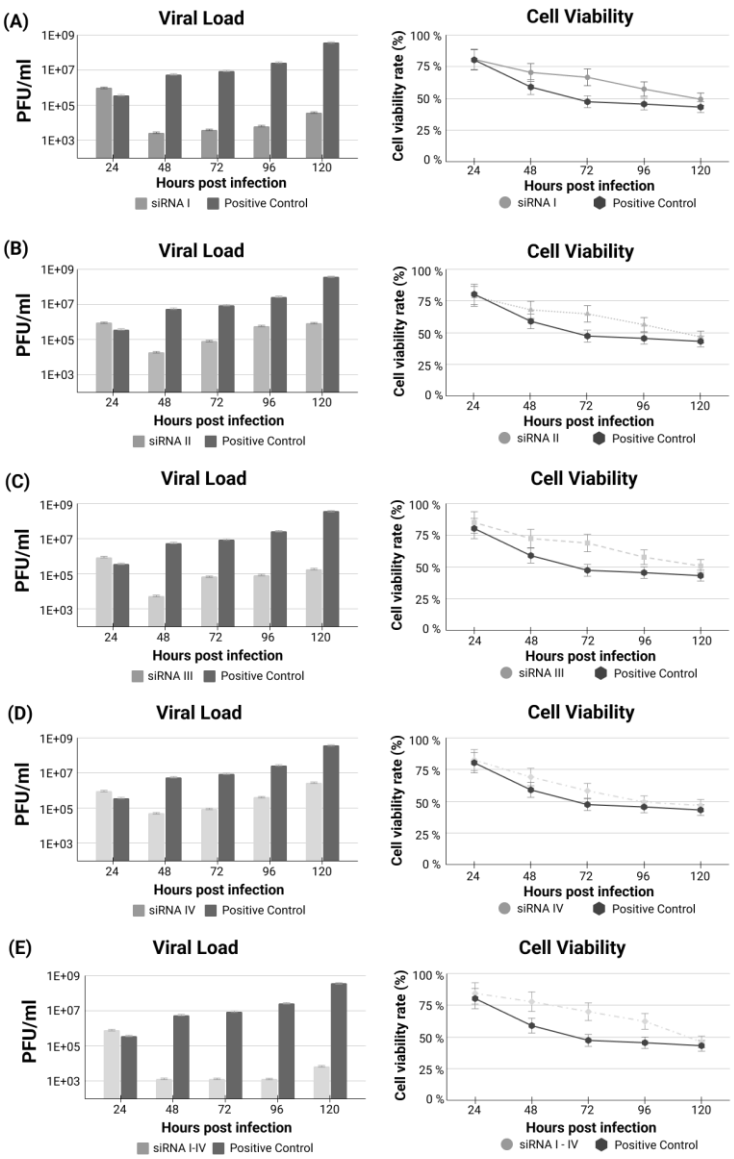

Figure 01. A-D Treatment with each siRNA individually (I to IV) after 24 hours of SARS-CoV-2 infection. **E** Analysis of viral load and cell viability after treatment using the cocktail containing the combination of siRNAs I to IV.

Nevertheless, this improvement in the efficacy due to using the siRNA combination, individual siRNA treatments, and the combination including the four siRNAs was highly efficient in provoking a marked decrease in viral load just before starting treatment and during the first three days but allowed a subtle regrowth of viral load observed at the fourth day, suggesting a deterioration in efficacy.

Addressing this hypothesis, an additional siRNA, siRNA-V, was incorporated into the experiments to block the hypothetical escape strategy of the virus.

A comparison of treatments using the combination of siRNA-I plus siRNA-III, the most individually efficient siRNAs, versus the combination of siRNA-I plus siRNA-III plus the addition of siRNA-V, the anti-escape siRNAs, is demonstrated (Figure 02).

The addition of siRNA-V prevents virus regrowth, keeping the viral load down throughout the experimental time. Interestingly, the inclusion of siRNA-V also improved cellular viability, strongly demonstrating the inhibition of the virus' escape mechanism (Figure 02).

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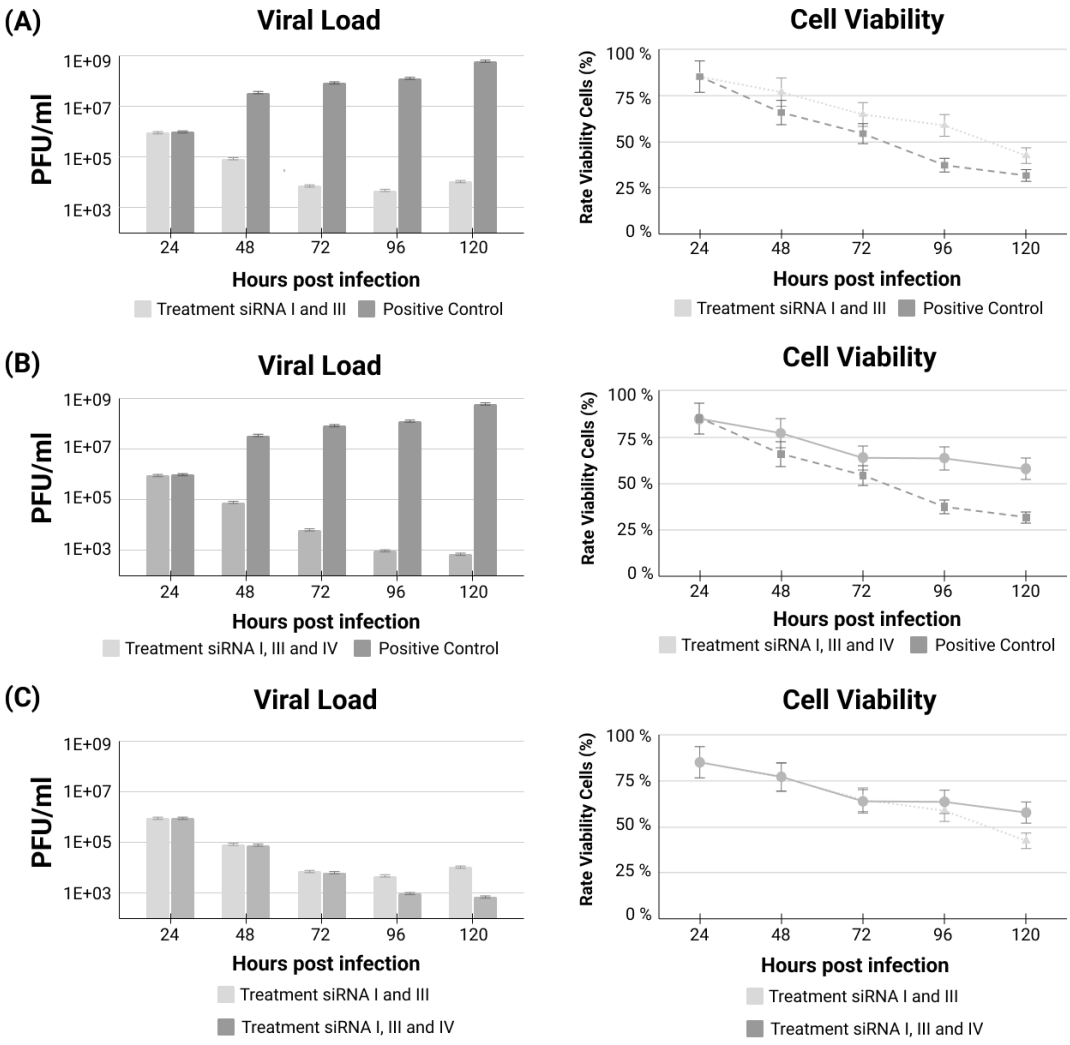

Figure 02. – Viral load and cell viability comparing untreated cells versus both the combination of siRNA-I plus siRNA-III treatment (A); the combination of siRNA-I plus siRNA-III plus the addition of the anti-escape siRNA-V treatment (B); and the comparison between the two treatment combinations (C).

4. Discussion

The emergence and continuation of the SARS-CoV-2 pandemic became a challenge for health care professionals to rapidly combat the spread of COVID-19. Effective treatments against COVID-19 are still scarce and without widespread availability, thus becoming a major public health problem [15]. The development of an efficient, short course, and widely available treatment strategy to prevent viral replication and disease progression represents a potential weapon to control the pandemic, together with available protective measures, vaccination and medical care [9,16].

Attempts using siRNA against viral infections have already been described in cases of human immunodeficiency virus, influenza virus, coxsackie B3, adenovirus, hepatitis B virus, hepatitis C virus, and to tackle SARS-CoV in various cellular and animal models [17], thus supporting the investigation of such a strategy to combat COVID-19 [9,17,18].

Consequently, a targeted therapy against SARS-CoV-2 using siRNAs was proposed and proved to be highly effective in reducing the viral load of infected non-transformed thyroid human cells and mainly safe, since those cells were not injured by the therapy and tended to recover from viral aggression, thus opening a path to proceeding to animal and human trials.

Aiming to achieve reliable siRNA therapy, the viral genome and human infection processes were studied in depth to determine the best targets to avoid effective viral

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replication and infectiousness potential. Additionally, a broad analysis of available SARS-CoV-2 genomes was also considered in the search for highly conserved non-mutated regions able to be targeted, without chances of having some virus variants capable of escaping from the selected siRNAs. Indeed, the chosen targets are present in all variants of interest that have already been sequenced, expanding the prospects for sustained successful clinical use. Other researchers also selected the same genes (1a and 1ab) as the best targets for siRNAs [19], although additional SARS-CoV-2 genes were also silenced by siRNA, achieving good results both in cell lines and in animal models [17]. Recently, siRNA treatment against SARS-CoV-2 was authorized for a clinical trial in Europe [20]because it achieved promising results in preclinical models [21].

After choosing the potential targets, a very rigorous *in silico* search for similar sequences in both humans and nonhuman genomic banks was performed, warranting a virtual "zero risk" for off-target events.

Compared to other siRNA experiments against SARS-CoV-2 infections carried out in nonhuman cells [22] and in transformed human cells [23], the developed nontransformed thyroid human cell model presents an important advantage, since it represents a SARS-CoV-2-susceptible normal human cell, allowing the investigation of the safety of the proposed treatment for normal human cells. In particular, off-target effects harming the normal human genome could be explored in an adequate model. Additionally, the effects of the infection and the potential protective benefits of the treatment could be investigated in a reliable model compared to the usually applied nonhuman and/or transformed cell models [24].

The proposed siRNAs were designed in-house to guarantee the agreed upon priorities, including high specificity, an almost null possibility of harming human RNAs, and not matching to any other organism's genome, in addition to the conventional requirements for stable and efficient siRNA construction protocols.

Another improvement of the suggested strategy of combined siRNAs was related choosing the best virus strand's targets for both replication and escape sequences.

SARS-CoV-2 is a positive single-strand RNA virus, and after gaining access to human cells through ACE2 (angiotensin-converting enzyme II) cell receptors, the viral replication process encompasses a few steps that are critical for selecting the best targets, as briefly described.

The positive RNA strand uses the cellular machinery to transcribe two of its main genes, known as ORF1a and ORF1ab, but does not duplicate the whole virus genome. The peptides resulting from this human machinery-assisted process form the virus replicase that allows virus self-replication [25].

This replicase provides duplication of the whole positive strand, resulting in a negative strand. The negative strand is the generator of the new positive strands and finally allows the multiplication of the virus inside the human cell, keeping the infection active, since lots of new viruses will leave that cell to infect other host's cells. The negative strand provides positive strand "products" by two different mechanisms: a negative strand is duplicated in a whole positive strand, the genomic replication, and a subgenomic replication process, where fragments of the positive strand, including specific structural genes, but not ORF1a and 1ab, are generated [26].

Looking at this rich scenario, many possibilities for addressing viral sequences can be realized, and the best targets for siRNA treatment remain under debate.

Our choices were influenced by some concepts and conveniences. We decided to focus on the ORF1a/1ab region of the positive strand, since transcription of these regions is the first step for virus persistence in human cells and is absolutely necessary to produce the virus replicase, which is indispensable for sustainable infection [18].

In contrast to most previous attempts to control SARS-CoV-2 infection using a unique siRNA [17,24], combinations of siRNA were selected. Initially, four siRNAs were specifically designed to bind complementary sequences found exclusively in the SARS-CoV-2 genome and presented in every clinically relevant published SARS-CoV-2 genome, including variants Alpha, Beta, Gamma, and Omicron. Conceptually, impeding virus

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replication because of the inactivation of ORF1a/1ab should control the infection and, actually, the proposed siRNAs were extremely effective, reducing viral loads both individually and in different combinations, as demonstrated.

Although provoking extraordinary decreases in viral load and allowing cellular recovery after infection, a few days after the initial siRNA treatment, a subtle increase in virus production seemed to occur, nevertheless in a small fraction, compared to nontreated controls.

A hypothesis of a virus's escape mechanism was considered, since extra doses of siRNAs failed to revert this phenomenon, and many viruses, including coronaviruses, have the capacity to escape siRNA attacks, as demonstrated in depth [6]. Accordingly, the virus might escape from siRNA by producing siRNA suppressors, so after being hit by the first dose of siRNAs, it counterattacks by increasing the production of siRNA suppressor sequences, making the new siRNA doses much less effective. Among putative virus escape strategies, the possibility of preventing the RNA inhibition silencing complex (RISC), the effective complex containing the siRNAs that bind and inactivate the target sequences function by the production of specific sequences has been discussed.

An extensive *in silico* investigation permitted the conception and construction of an extra siRNA molecule designed against this putative anti-siRNA sequence, the incorporation of which in the siRNA combination blocked the assumed escape mechanism. Nevertheless, other escape mechanisms have been reported, such as modifications in ribosomes of infected cells allowing translation of temporary mutations [27,28]. The strategy of adding siRNA-V, the "anti-siRNA suppressor bullet", seemed to be correct, since it actually counterattacked the putative RISC-based virus escape strategy, keeping the virus load much lower even after some days of treatment.

Another strategic decision was that, instead of waiting for the supposed siRNA suppressor escape mechanism, the anti-escape siRNA was preliminarily launched at the onset of treatment as chess move anticipation.

Importantly, the choice for targeting the anti-siRNA sequence needed to be at a subgenomic region, since the *in silico* prediction pointed to a region away from ORF1ab. Considering that many positive-strand subgenomic fragments are produced, if the positive-strand target is selected, a tremendous reduction in efficacy should be anticipated due to the great number of targets to be inactivated. Instead of targeting those multiple fragments, our choice fell at the negative strand, the one that originates all positive subgenomic fragments. The selected siRNAs (protected by patents) showed immense potential in consistently reducing viral load without harming human cells.

5. Conclusions

The combination of siRNAs I, III and V, addressing both virus replication and the siRNA suppressor escape mechanism, seems to be the best formulation for clinical investigation due to safety, efficiency, and prolonged sustained activity, imposing a checkmate to SARS-CoV-2.

These extraordinary preliminary results open the path to investigate this strategy in animal models and, subsequently, in human clinical trials, aiming to provide the potential benefits of this innovative approach against SARS-CoV-2 and COVID-19 and even pave the way for siRNA therapy applicability in several other infectious and chronic diseases.

Supplementary Materials: The following supporting information can be downloaded at:

The data presented in this study are openly available in FigShare at https://doi.org/10.6084/m9.figshare.20415957, Figure A1: Experimental design for the treatment of IMR-90 cells infected with SARS-CoV-2.

The data presented in this study are openly available in FigShare at https://doi.org/10.6084/m9.figshare.20415966, Figure A2: **(A) I** Non-transformed thyroid cells after a period of 120 hours in culture (uninfected controls). **(A) II** IMR-90 cells 120 hpi by SARS-Cov-2 **(B)**

Daily viral load quantification according to the number of produced plaques (PFU/ml). (C) Cellular viability in infected and control cells (MOCK).
The data presented in this study are openly available in FigShare at https://doi.org/10.6084/m9.figshare.20415963, Figure A3: **(A)** Comparison between cell viability, mitochondrial viability and viral load in IMR-90 cells infected with SARS-CoV-2. **(B)** Comparison between results of caspase expression and cytopathic effect (CPE) in IMR-90 cells infected with SARS-CoV-2.
The data presented in this study are openly available in FigShare at https://doi.org/10.6084/m9.figshare.20415960, Figure A4: Cell toxicity rate for siRNA exposure relating to unexposed cells.

Author Contributions: Conceptualization Casseb, S.M.M.; Khayat, A.S.; Santos, S.E.B.D.; da Costa Vasconcelos, P.; de Assumpção, P. Data curation de Souza, J.; de Oliveira, E. Methodology, de Souza, J; Oliveira, E and Casseb, S.M.M. Writing—review & editing Casseb, S.M.M.; Khayat, A.S.; de Souza, J.; de Oliveira, E.; Santos, S.E.B.D.; da Costa Vasconcelos, P.; de Assumpção, P . All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

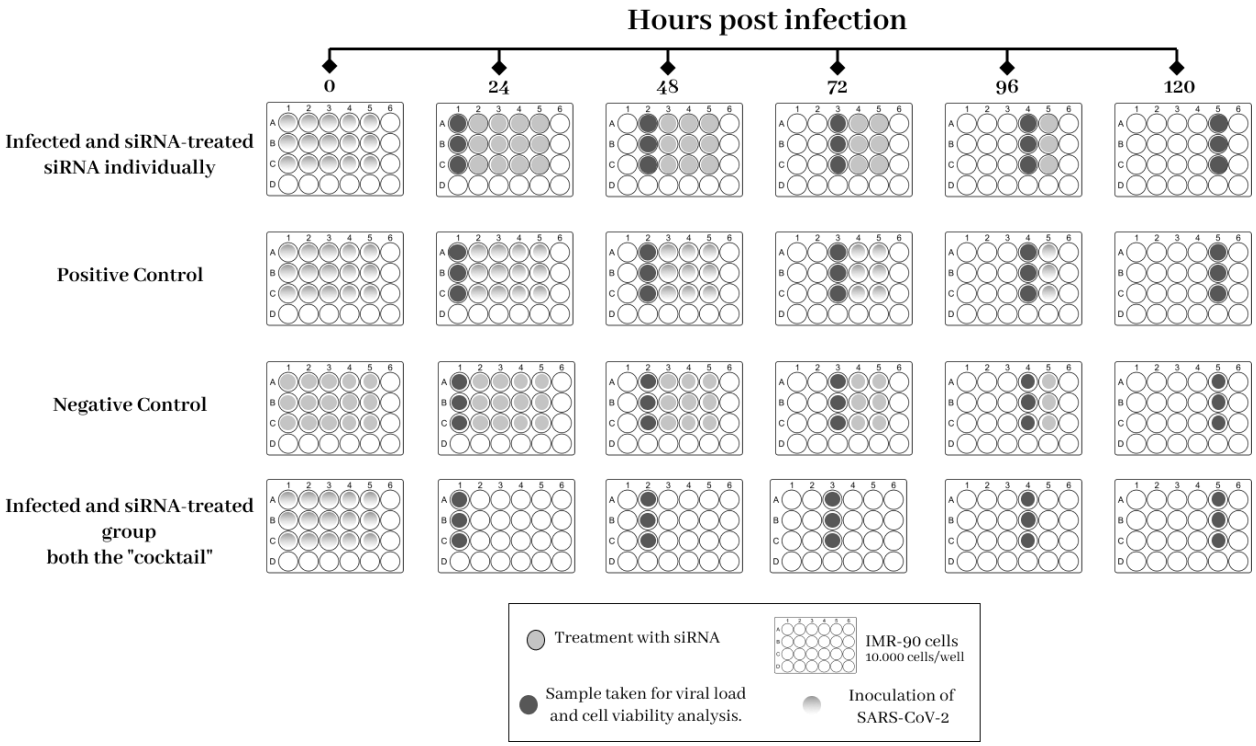

Figure A1. - Experimental design for the treatment of IMR-90 cells infected with SARS-CoV-2.

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Appendix B

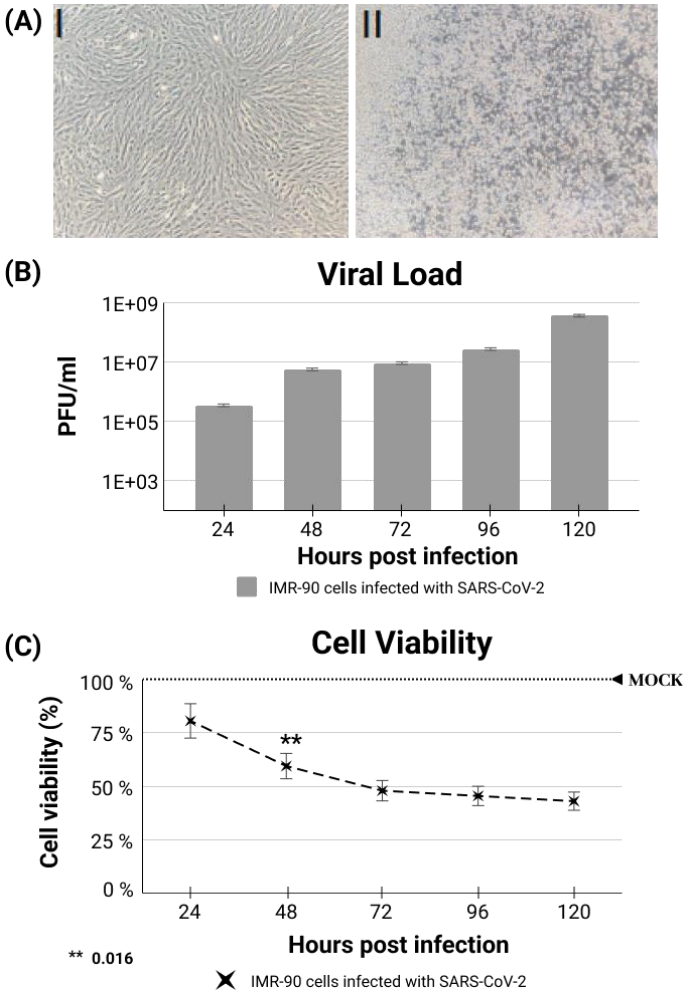

Figure A2. - (A) I Non-transformed thyroid cells after a period of 120 hours in culture (uninfected controls). **(A) II** IMR-90 cells 120 hpi by SARS-Cov-2 **(B)** Daily viral load quantification according to the number of produced plaques (PFU/ml). (C) Cellular viability in infected and control cells (MOCK).

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Appendix C

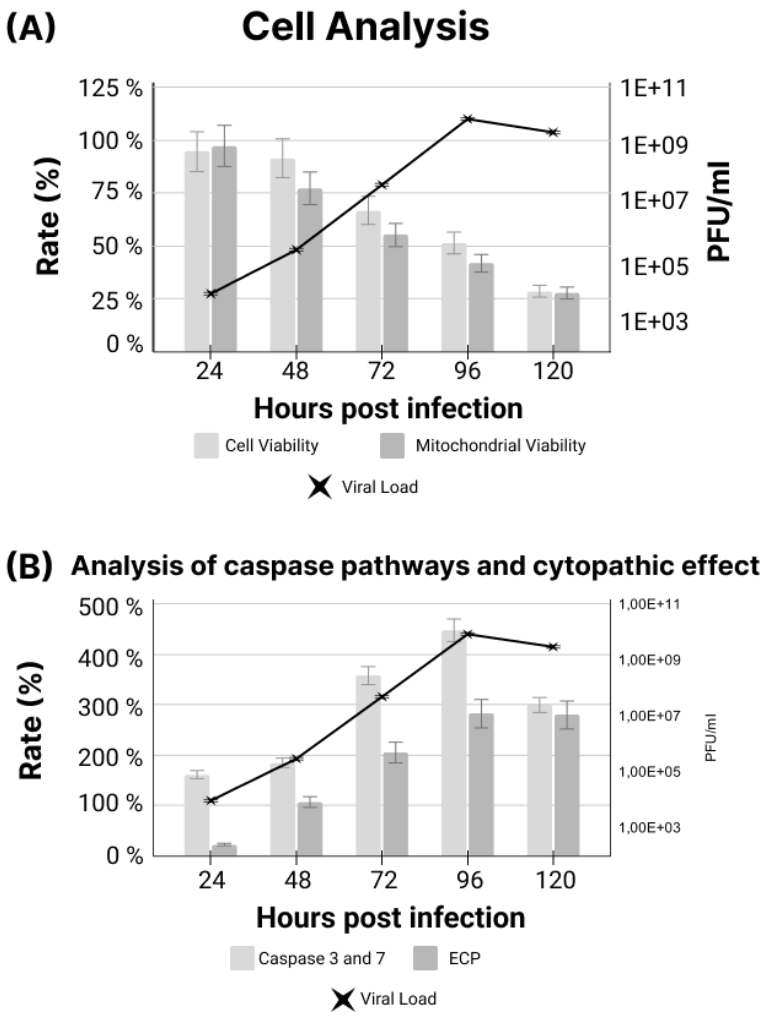

Figure A3. - (A) Comparison between cell viability, mitochondrial viability and viral load in IMR-90 cells infected with SARS-CoV-2. **(B)** Comparison between results of caspase expression and cytopathic effect (CPE) in IMR-90 cells infected with SARS-CoV-2.

Appendix D

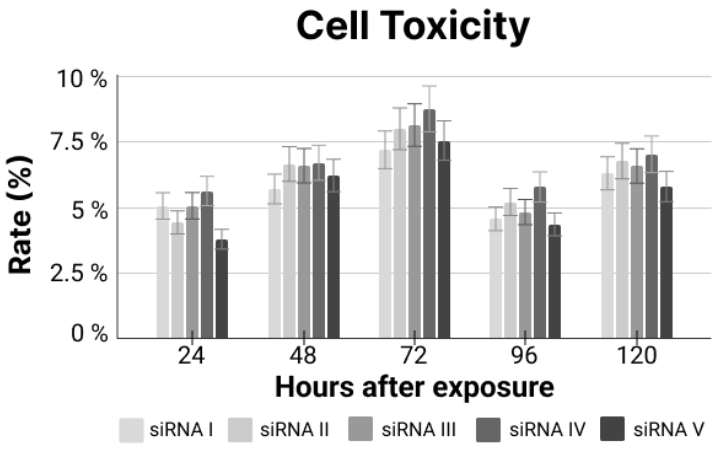

Figure A4. - Cell toxicity rate for siRNA exposure relating to unexposed cells.

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