Highly potent antisense oligonucleotides: locked nucleic acid gapmers targeting the SARS-CoV-2 RNA genome

Dauksaite, V.; Tas, A.; Wachowius, F.; Spruit, A.; Hemert, M.J. van; Snijder, E.J.; ... ; Zonneveld, A.J. van

NUCLEIC ACID THERAPEUTICS
Volume 33, Number 6, 2023

DOI: 10.1089/nat.2023.0012

Highly Potent Antisense Oligonucleotides Locked Nucleic Acid Gapmers Targeting the SARS-CoV-2 RNA Genome

Vita Dauksaite,[1,*] Ali Tas,[2] Falk Wachowius,[3] Anouk Spruit,[1,†] Martijn J. van Hemert,[2] Eric J. Snijder,[2] Eric P. van der Veer,[3] and Anton Jan van Zonneveld[1]

The severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has caused the current worldwide pandemic and the associated coronavirus disease 2019 with potentially lethal outcome. Although effective vaccines strongly contributed to reduce disease severity, establishing a toolbox to control current and newly emerging coronaviruses of epidemic concern requires the development of novel therapeutic compounds, to treat severely infected individuals and to prevent virus transmission. Here we present a therapeutic strategy targeting the SARS-CoV-2 RNA genome using antisense oligonucleotides (ASOs). We demonstrate that selected locked nucleic acid gapmers have the potency to reduce the *in vitro* intracellular viral load by up to 96%. Our promising results strongly support the case for further development of our preselected ASOs as therapeutic or prophylactic antiviral agents.

Keywords: antisense oligonucleotides, LNA gapmers, positive-sense RNA virus, coronavirus, SARS-CoV-2, therapeutic oligonucleotides

Introduction

Mankind is currently dealing with the third outbreak in 20 years of an emerging human-infecting coronavirus, the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) [1]. The pandemic was partially contained due to the successful development of vaccines, especially the newly emerged messenger RNA (mRNA)-based vaccines [2]. However, antiviral drugs are and will be needed to complement vaccination in the clinical arena, for example, for patients with severe chronic infections, to serve as prophylactics for high-risk groups, or to control future outbreaks of novel coronaviruses.

Multiple attempts to repurpose existing drugs resulted in few candidates that are currently used in clinics (eg, Paxlovid, Molnupiravir [3]). Other therapeutic strategies have been developed particularly aiming to reduce viral transmission, including ''miniproteins'' [4], lipopeptides [5], antibody cocktails [6], or small-molecule drugs that target viral components [7].

We took a parallel approach to develop antisense oligonucleotides (ASOs) that specifically target SARS-CoV-2 viral RNA, aiming to block viral replication. SARS-CoV-2 is a positive-sense single-stranded RNA virus with a genome of ~30 kb [8] and well-described structural and functional RNA elements [9–11]. Given the structural architecture of the SARS-CoV-2 RNA genome, we specifically selected (un)structured elements that can serve as ASO binding site and that were known to have functional roles in viral replication (Fig. 1A) [12–14].

We designed multiple viral RNA-targeting ASOs of different chemistries (locked nucleic acid gapmers [LNA

[1]Einthoven Laboratory for Vascular and Regenerative Medicine, Department of Internal Medicine (Nephrology), Leiden University Medical Centre, Leiden, The Netherlands.
[2]Department of Medical Microbiology, Leiden University Medical Centre, Leiden, The Netherlands.
[3]Hybridize Therapeutics, Oegstgeest, The Netherlands.
**Current address:* Department of Human Genetics, Leiden University Medical Centre, Leiden, The Netherlands.
[†]*Current address:* Dutch Center for RNA Therapeutics, The Netherlands.

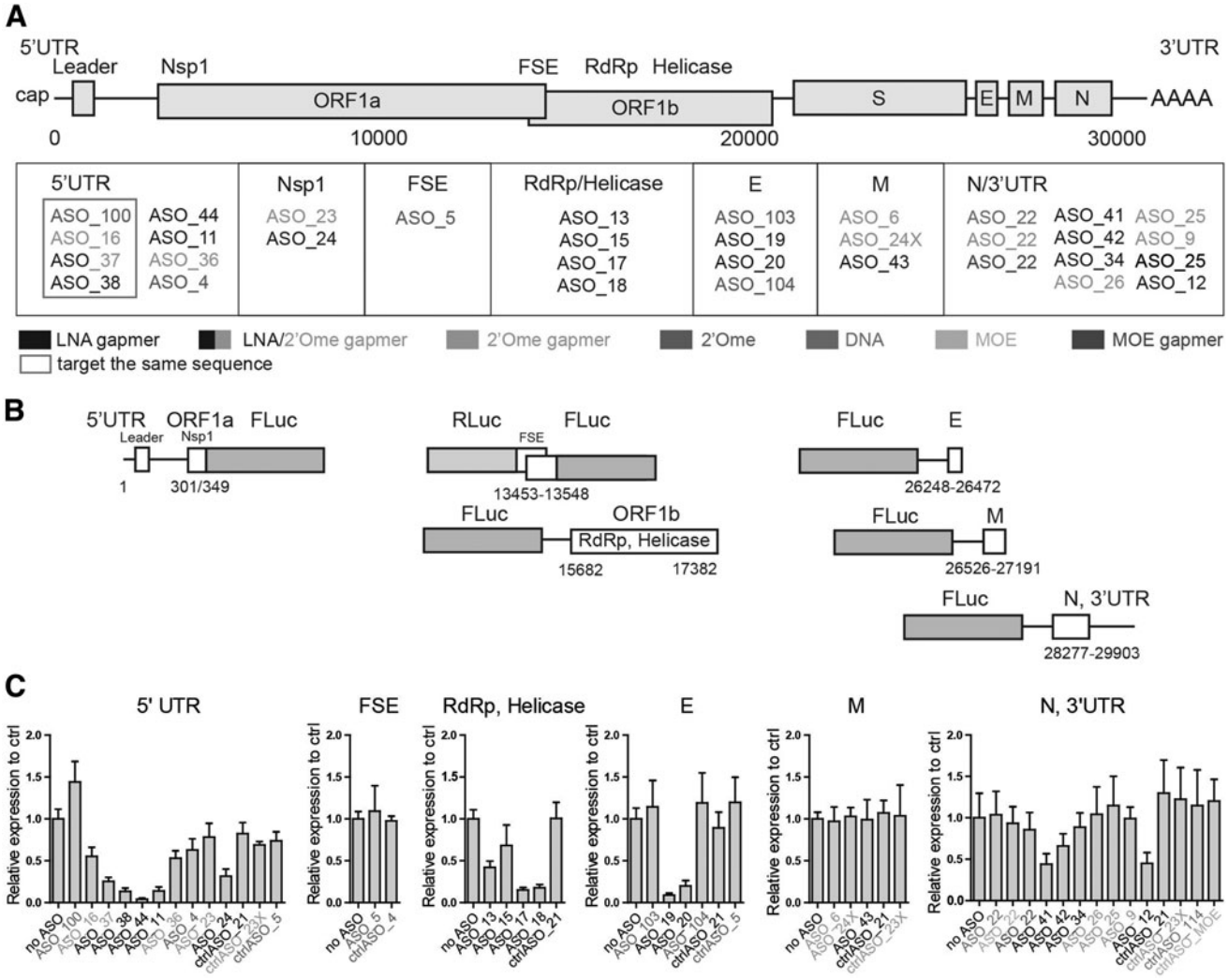

FIG. 1. *In vitro* screening of ASOs targeting the SARS-CoV-2 genome in a cell-based assay. **(A)** Organization of the SARS-CoV-2 RNA genome showing the 5′-UTR, the two known ORFs (ORF1a) and (ORF1b), major subgenomic RNAs, and the 3′-UTR. Scale marker for the 30 kb genome is indicated. ASOs below are aligned according to the genomic regions they target. Legend to the ASO chemistry is indicated below. **(B)** Schematic representation of luciferase reporter constructs used to screen ASOs in the *in vitro* assays. SARS-CoV-2 genomic regions, included in constructs, are indicated below. **(C)** Vero E6 cells were transiently transfected with the reporter construct harboring the SARS-CoV-2 genomic regions, the control constructs and treated with the indicated ASO. Luciferase readings were assayed 30 h later. Inhibitory effect was calculated as the ratio of relative luminescence units in the presence of a specific concentration of ASO and the relative luminescence units in the absence of ASO (no ASO) and corrected for background luminescence. Each ASO in a particular experiment was assayed in triplicate. Data are means (±) SD from at least three independent experiments. ASOs, antisense oligonucleotides; FSE, ribosomal frameshift element; LNA, locked nucleic acid; MOE, 2′-O-methoxyethyl; ORFs, open reading frames; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2; SD, standard deviation; UTR, untranslated region.

gapmers] [DNA ASOs with RNA-like segments on both sides], LNA-2'Ome gapmers, 2'Ome gapmers, 2'Omes, 2′-O-methoxyethyl [MOEs], MOE gapmers). Gapmers [15] are powerful tools for mRNA loss-of-function studies as they catalyze the RNase H-dependent degradation of complementary RNA targets, whereas RNA ASOs are expected to interfere through sterical hindrance [16]. The LNA gapmers (containing the LNA flanking regions) are presumed to be superior to other gapmers, as the LNA region increases target binding affinity and confers nuclease resistance [15].

Here we present the selection and *in vitro* evaluation of new antiviral ASOs against SARS-CoV-2, with the potential of developing a new therapeutic appproach and prevention options for future emerging coronaviruses of pandemic concern. Our findings are in line with much further developed LNA ASO therapeutics [17] and with numerous siRNA therapeutics developed against SARS-CoV-2 [18–22].

Materials and Methods

Molecular cloning

Firefly luciferase reporter constructs were generated by inserting appropriate viral genome segments into the pCI-neo-FLuc (exact nucleotide positions indicated in Figs. 1B and 3A).

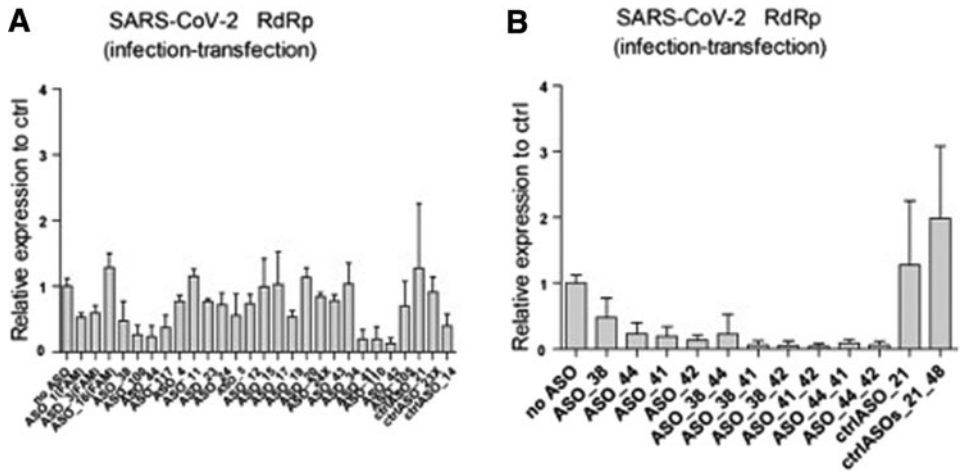

FIG. 2. Selected ASOs are potent in reducing intracellular viral RNA levels. **(A)** The most effective ASOs targeting each viral region have been further tested in transient transfections of the Vero E6 cells using the live SARS-CoV-2 (infection first, then ASO treatment [transfection]). Viral loads were detected through qRT-PCR. **(B)** In part A preselected lead candidates were further screened separately or in combination (synergy effect) in Vero E6 cells using the live SARS-CoV-2 and first infection then transfection approach. The percentage of intracellular viral load reduction is shown relative to the control (no ASO treatment). Data are means ± SD from three independent experiments. qRT-PCR, real-time reverse transcription PCR.

Antisense oligonucleotides

All ASOs (containing fully modified phosphorothioate backbones) were produced by Eurogentec. ASO sequences and target locations in the SARS-CoV-2 genome are specified in Supplementary Table S1 and depicted schematically in Fig. 1A.

Cell culture and transfections

Vero E6 cells were cultured as described [23]. For luciferase assays, cells were Lipofectamine transfected with the reporter, control plasmid, and ASO, readings were performed 30 h later (SpectraMax i3x; Molecular Devices). To assess

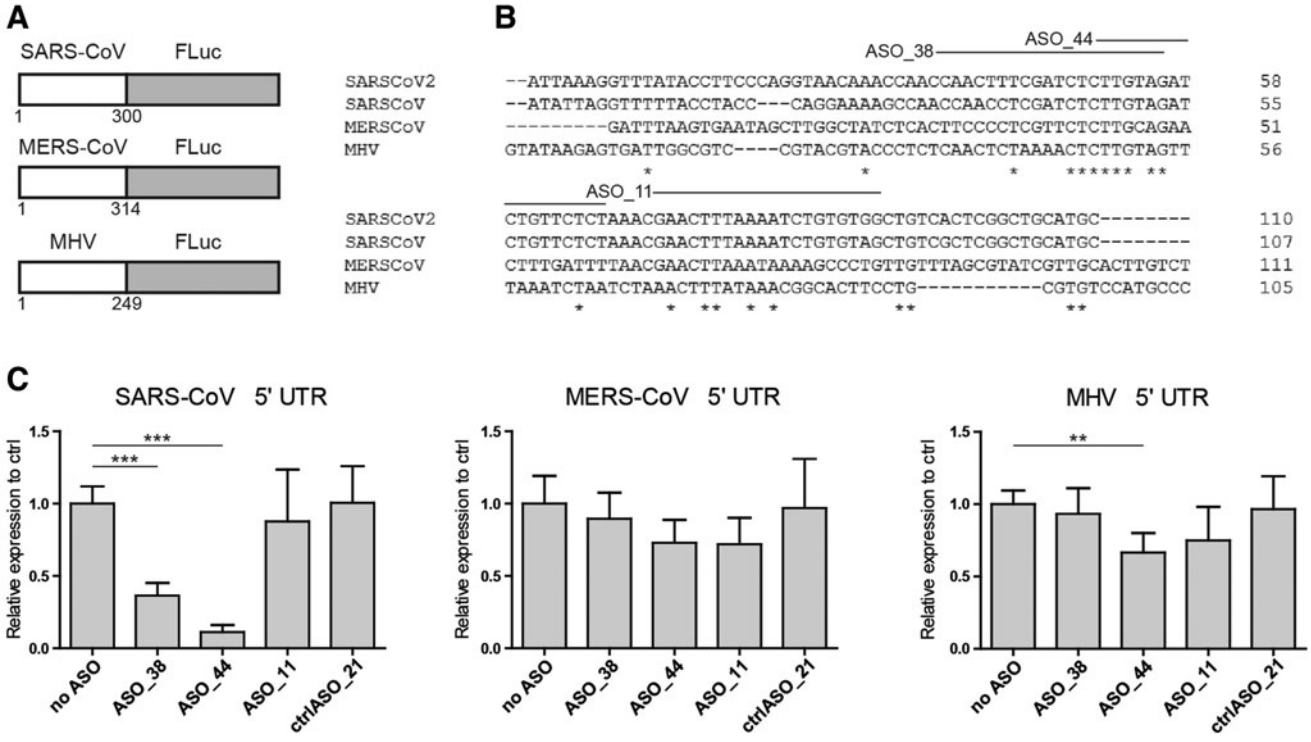

FIG. 3. *In vitro* screening of SARS-CoV-2 genome-targeting ASOs against the other SARS-related betacoronaviruses. **(A)** Schematic representation of the luciferase reporter constructs harboring the 5′ UTR regions of SARS-CoV, MERS-CoV, and MHV. **(B)** Clustal-performed alignment of the conserved nucleotides is indicated with *. **(C)** Vero E6 cells were transfected with the reporter constructs where SARS-CoV-2 genomic sequences are fussed to the Firefly luciferase gene and treated with ASO. Luciferase readings were assayed 30 h later. Group comparisons were analyzed by one-way ANOVA and Bonferoni post-test. *P* values were considered statistically significant as follows: $^{**}P < 0.01$; $^{***}P < 0.001$. ANOVA, analysis of variance; MERS-CoV, Middle East respiratory syndrome coronavirus; MHV, mouse hepatitis virus.

antiviral activity, cells were infected with SARS-CoV-2 for 1 h at 37°C with 12,000 Plaque forming unit (PFU)/well, followed by a 16-h ASO treatment. Multiplex real-time reverse transcription PCR (qRT-PCR) analysis was performed using Taqman probes against the SARS-CoV-2 RdRp gene, whereas the mRNA of the phosphoglycerate kinase 1 (PGK1) housekeeping gene was used for normalization.

Results and Discussion

To facilitate ASO selection, we conducted the initial functional evaluation in Vero E6 cells transfected with reporter gene constructs expressing Firefly luciferase in combination with specific SARS-CoV-2 genome segments. The viral sequences included in the reporter mRNAs are depicted in Fig. 1B. Each reporter construct tested resulted in the selection of ASOs reducing the level of luciferase in comparison with no ASO/ctrlASO (scrambled sequence) controls (Fig. 1C). At this time point, different ASO chemistries were tested and the LNA gapmers seemed to be more potent than the LNA-2'Ome or 2'Ome gapmers.

To exclude potential effects resulting from ASO toxicity, cell viability assays were performed in parallel with this experiment (Supplementary Fig. S5). Testing the most potent ASOs targeting the SARS-CoV-2 5′ untranslated region (UTR) in the same assay set-up in Calu-3 cells (a human lung cancer cell line) resulted in a comparable reduction of luciferase activity (Supplementary Fig. S1). SARS-CoV-2 5′ UTR-targeting lead candidate ASOs were tested to select the most potent gapmer sequence. The arrangement of 10–13 nt central DNA strand flanked by the four LNA nucleotides from the 5′- and the 3′-ends was the most optimal (Supplementary Fig. S2).

To evaluate the level of interference with virus replication of the ASOs selected in the reporter assays, we performed experiments using SARS-CoV-2-infected Vero E6 cells. Initially we used the commonly used experimental set-up in which cells are first pretreated with ASOs before being infected with SARS-CoV-2 (Supplementary Fig. S3). Next we switched to experimental set-up where Vero E6 cells are first infected with SARS-CoV-2 for 1 h, followed by virus removal from the medium and the ASO treatment of the infected cells for 16 h. Intracellular RNA was assayed for the reduction of viral RNA loads in qRT-PCR using primers targeting the viral RdRp and E protein genes.

Using both approaches, the 5′ UTR-targeting ASOs 38 and 44 as well as the N gene-targeting ASOs 41 and 42 were identified as the most potent, leading to a 53%–87% reduction of the intracellular viral RNA load when used alone (depicted in Fig. 2A; data using primers targeting the viral E gene are depicted in Supplementary Fig. S4A). Using any combination of these ASOs, their potency was retained, and the effect was even strengthened, leading up to a 96% reduction of the intracellular viral RNA load (Fig. 2B; Supplementary Fig. S4B). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay demonstrated no effect of the ASOs used on cell viability.

Additional reporter constructs have been generated to assay the potency of lead candidate ASOs against highly conserved sequences from other betacoronaviruses (Fig. 3B). To evaluate the potential for broad-spectrum activity, the lead candidate ASOs targeting the SARS-CoV-2 5′ UTR region were tested using constructs carrying 5′ UTR sequences from SARS-CoV, Middle East respiratory syndrome coronavirus (MERS-CoV), and mouse hepatitis virus (MHV). Figure 3C shows that lead candidate ASOs 38 and 44 are most active against SARS-CoV sequences and in addition ASO 44 showed potency against MHV sequence, but no significant effect was obtained against the MERS-CoV sequence.

Based on the *in vitro* results presented here, we expect that ASOs may be effective in reducing virus replication *in vivo*. Likewise, our *in vitro* data suggest that ASOs might be effective against other emerging coronaviruses of pandemic concern. In addition, testing of already identified highly conserved weakly structured regions within viral RNA genomes might aid the development of antiviral ASO therapeutics [12].

Work still needs to be done to define the best administration method (intranasal or systemic) and the uptake in the upper and lower respiratory tract, in the systemic circulation and organs. Selected ASOs need to be evaluated for the efficacy *in vivo*, stability and toxicity. Operating by a different mechanism, ASOs could be envisioned as additives to the existing/in development antivirals for intranasal prophylactic approach, preventing viral transmission.

Author Disclosure Statement

The authors declare no conflict of interest.

Funding Information

This study was supported by funding from Health Holland, Grant TKI-LSHM20039 ''First line of Defence.''

Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
Supplementary Figure S4
Supplementary Figure S5
Supplementary Table S1

References

1. Zhou P, XL Yang, XG Wang, B Hu, L Zhang, W Zhang, HR Si, Y Zhu, B Li, *et al.* (2020). A pneumonia outbreak associated with a new coronavirus of probable bat origin. Nature 579:270–273.
2. Cosentino M and F Marino. (2022). Understanding the pharmacology of COVID-19 mRNA vaccines: Playing dice with the spike? Int J Mol Sci 23:10881.
3. EMA. (2021). EMA issues advice on the use of Paxlovid (PF-07321332 and ritonavir) and Lagevrio for the treatment of COVID-19. https://wwwemaeuropaeu/en/news/ema-issues-advice-use-paxlovid-pf-07321332-ritonavir-treatment-covid-19-rolling-review-starts; https://wwwemaeuropaeu/en/news/ema-issues-advice-use-lagevrio-molnupiravir-treatment-covid-19.
4. Cao L, I Goreshnik, B Coventry, JB Case, L Miller, L Kozodoy, RE Chen, L Carter, AC Walls, *et al.* (2020). De novo design of picomolar SARS-CoV-2 miniprotein inhibitors. Science 370:426–431.
5. de Vries RD, KS Schmitz, FT Bovier, C Predella, J Khao, D Noack, BL Haagmans, S Herfst, KN Stearns, *et al.* (2021). Intranasal fusion inhibitory lipopeptide prevents direct-contact SARS-CoV-2 transmission in ferrets. Science 371:1379–1382.

6. Weinreich DM, S Sivapalasingam, T Norton, S Ali, H Gao, R Bhore, J Xiao, AT Hooper, JD Hamilton, *et al.* (2021). REGEN-COV antibody combination and outcomes in outpatients with COVID-19. N Engl J Med 385:e81.
7. Hermann T. (2016). Small molecules targeting viral RNA. Wiley Interdiscip Rev RNA 7:726–743.
8. de Vries AAF. (2020). SARS-CoV-2/COVID-19: a primer for cardiologists. Neth Heart J 28:366–383.
9. Yang D and JL Leibowitz. (2015). The structure and functions of coronavirus genomic 3' and 5' ends. Virus Res 206:120–133.
10. Madhugiri R, N Karl, D Petersen, K Lamkiewicz, M Fricke, U Wend, R Scheuer, M Marz and J Ziebuhr. (2018). Structural and functional conservation of cis-acting RNA elements in coronavirus 5'-terminal genome regions. Virology 517:44–55.
11. Madhugiri R, M Fricke, M Marz and J Ziebuhr. (2016). Coronavirus cis-Acting RNA Elements. Adv Virus Res 96: 127–163.
12. Sanders W, EJ Fritch, EA Madden, RL Graham, HA Vincent, MT Heise, RS Baric and NJ Moorman. (2020). Comparative analyses of coronavirus genomic RNA structure reveals conservation in 4 SARS-like coronaviruses. bioRxiv. doi: 10.1101/2020.06.15.153197. Preprint.
13. Manfredonia I, C Nithin, A Ponce-Salvatierra, P Ghosh, TK Wirecki, T Marinus, NS Ogando, EJ Snijder, MJ van Hemert, JM Bujnicki and D Incarnato. (2020). Genome-wide mapping of SARS-CoV-2 RNA structures identifies therapeutically-relevant elements. Nucleic Acids Res 48: 12436–12452.
14. Huston NC, H Wan, MS Strine, R de Cesaris Araujo Tavares, CB Wilen and AM Pyle. (2021). Comprehensive in vivo secondary structure of the SARS-CoV-2 genome reveals novel regulatory motifs and mechanisms. Mol Cell 81:584.e5–598.e5.
15. Yokota T and R Maruyama. (2020). *Gapmers: Methods and Protocols. Methods in Molecular Biology, Volume 2176*. Humana: New York, NY.
16. Kole R, AR Krainer and S Altman. (2012). RNA therapeutics: beyond RNA interference and antisense oligonucleotides. Nat Rev Drug Discov 11:125–140.
17. Zhu C, JY Lee, JZ Woo, L Xu, X Nguyenla, LH Yamashiro, F Ji, SB Biering, E Van Dis, *et al.* (2022). An intranasal ASO therapeutic targeting SARS-CoV-2. Nat Commun 13:4503.
18. Khaitov M, A Nikonova, I Shilovskiy, K Kozhikhova, I Kofiadi, L Vishnyakova, A Nikolskii, P Gattinger, V Kovchina, *et al.* (2021). Silencing of SARS-CoV-2 with modified siRNA-peptide dendrimer formulation. Allergy 76:2840–2854.
19. Idris A, A Davis, A Supramaniam, D Acharya, G Kelly, Y Tayyar, N West, P Zhang, CLD McMillan, *et al.* (2021). A SARS-CoV-2 targeted siRNA-nanoparticle therapy for COVID-19. Mol Ther 29:2219–2226.
20. Hariharan VN, M Shin, CW Chang, D O'Reilly, A Biscans, K Yamada, Z Guo, M Somasundaran, Q Tang, *et al.* (2023). Divalent siRNAs are bioavailable in the lung and efficiently block SARS-CoV-2 infection. Proc Natl Acad Sci U S A 120:e2219523120.
21. Becker J, ML Stanifer, SR Leist, B Stolp, O Maiakovska, A West, E Wiedtke, K Borner, A Ghanem, *et al.* (2022). Ex vivo and in vivo suppression of SARS-CoV-2 with combinatorial AAV/RNAi expression vectors. Mol Ther 30:2005–2023.
22. Ambike S, CC Cheng, M Feuerherd, S Velkov, D Baldassi, SQ Afridi, D Porras-Gonzalez, X Wei, P Hagen, *et al.* (2022). Targeting genomic SARS-CoV-2 RNA with siRNAs allows efficient inhibition of viral replication and spread. Nucleic Acids Res 50:333–349.
23. Morandi E, I Manfredonia, LM Simon, F Anselmi, MJ van Hemert, S Oliviero and D Incarnato. (2021). Genome-scale deconvolution of RNA structure ensembles. Nat Methods 18:249–252.

Address correspondence to:
Prof. Dr. Anton Jan van Zonneveld
Einthoven Laboratory for Vascular and Regenerative Medicine
Department of Internal Medicine (Nephrology)
Leiden University Medical Center
Albinusdreef 2
Leiden 2333 ZA
The Netherlands

E-mail: a.j.van_zonneveld@lumc.nl

Received for publication March 9, 2023; accepted after revision August 11, 2023; Published Online: September 29, 2023.