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Discovery of Highly Potent Oligonucleotides Targeting SARS-CoV-2

ALIGOS THERAPEUTICS

Abstract 196

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Background

Development of therapeutic agents for the treatment of COVID-19 viral infection using short interfering RNA (siRNA) and antisense oligonucleotide (ASO) exemplifies a powerful new means to combat the ongoing COVID-19 pandemic. While ASO's might have the advantage of being fast acting, siRNAs demonstrate a longer duration of action and could potentially be developed as prophylactic agents. In this study, we aimed to evaluate specific binding to and silencing of SARS-CoV-2 mRNA by identifying the most effective siRNA's and ASO's from a panel of in silico designed oligonucleotides using an in vitro screening system.

Methods

190 siRNAs and 130 ASOs were designed using our in-house bioinformatics protocol, selecting for highly conserved SARS-CoV-2 target regions with oligonucleotides that had reduced risk of off-target activity; these were synthesized with multiple stabilizing chemistry designs. The in vitro screening system utilized a psiCHECK-2 vector overexpressing SARS-CoV-2 genome fragments fused with the Renilla luciferase reporter gene; firefly luciferase was used as intra-plasmid control. Assessment of decreases in Renilla luciferase activity allows for the detection of in vitro activity[1] of the oligonucleotides. The antiviral activity[2] of the most potent oligonucleotides was evaluated using A549-hACE-2 cells, infected with nanoluciferase-tagged SARS-CoV-2 virus for 24 hrs. For safety, lead oligonucleotides were tested for their potential to induce apoptosis or cytotoxicity.

Results

1. In vitro activity of siRNAs

We identified 14 unique target sequences clustered in 4 genome regions (depicted as orange arrows and blue boxes in Figure 1). These target sequences are hit by 2-4 siRNAs with different chemistries or lengths displaying EC_{50} values < 1.5 nM and maximum inhibition of reporter expression greater than/equal to 70% in the primary psiCHECK-2 luciferase reporter screen. One siRNA per target site was chosen for follow-up in the "live" virus assay. Assessment of antiviral activity in the SARS-CoV-2 viral infection assay, resulted in the identification of two highly effective siRNAs that significantly reduced virus replication with inhibition above 90% at 24 hrs and displaying an EC_{50} of < 0.5 nM (Figure 3). Addition of a phosphate mimic to the 5′ end of the antisense strand in combination with other stabilizing chemistries resulted in highly optimized siRNAs with enhanced antiviral capacity and EC_{50} values of ~20 pM for the two lead siRNAs. Binding sites of both siRNAs are outside of the viral Spike coding region. Lead siRNAs do not induce cytotoxicity as measured by Celltiter-Glo® at concentrations up to 500 nM in COS-7 and A549 cells. Based on bioinformatics analysis, both siRNAs retain activity against SARS-CoV-2 variants alpha (B.1.1.7), beta (B.1.351), gamma (P.1) and delta (B.1.617.2), omicron (B.1.1.529) as well as SARS-CoV-1 and MERS. Their binding sites do not overlap with signature mutations in SARS-CoV-2 variants of concern (downloaded from https://cov-lineages.org/ and reference pre-publications).

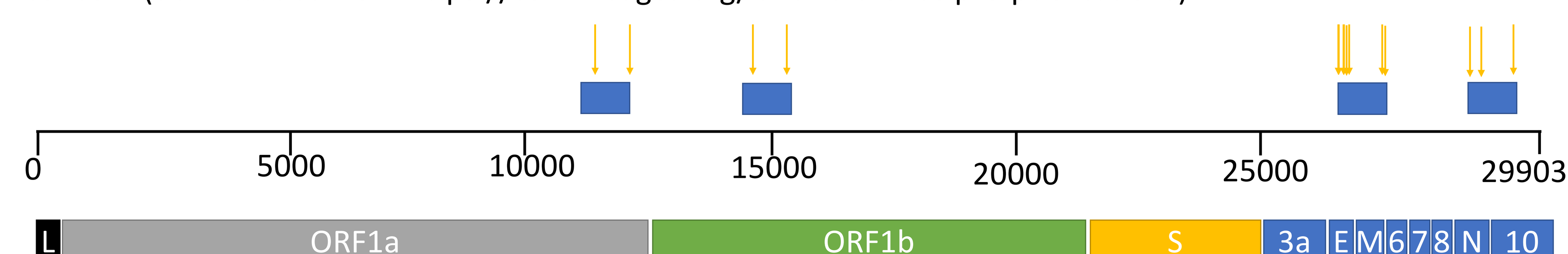


Figure 1: SARS-CoV-2 genome spanning 14 unique target sequences identified by initial screen

Hits from the psiCHECK-2 plasmid luciferase reporter assay were confirmed in a "live" virus assay

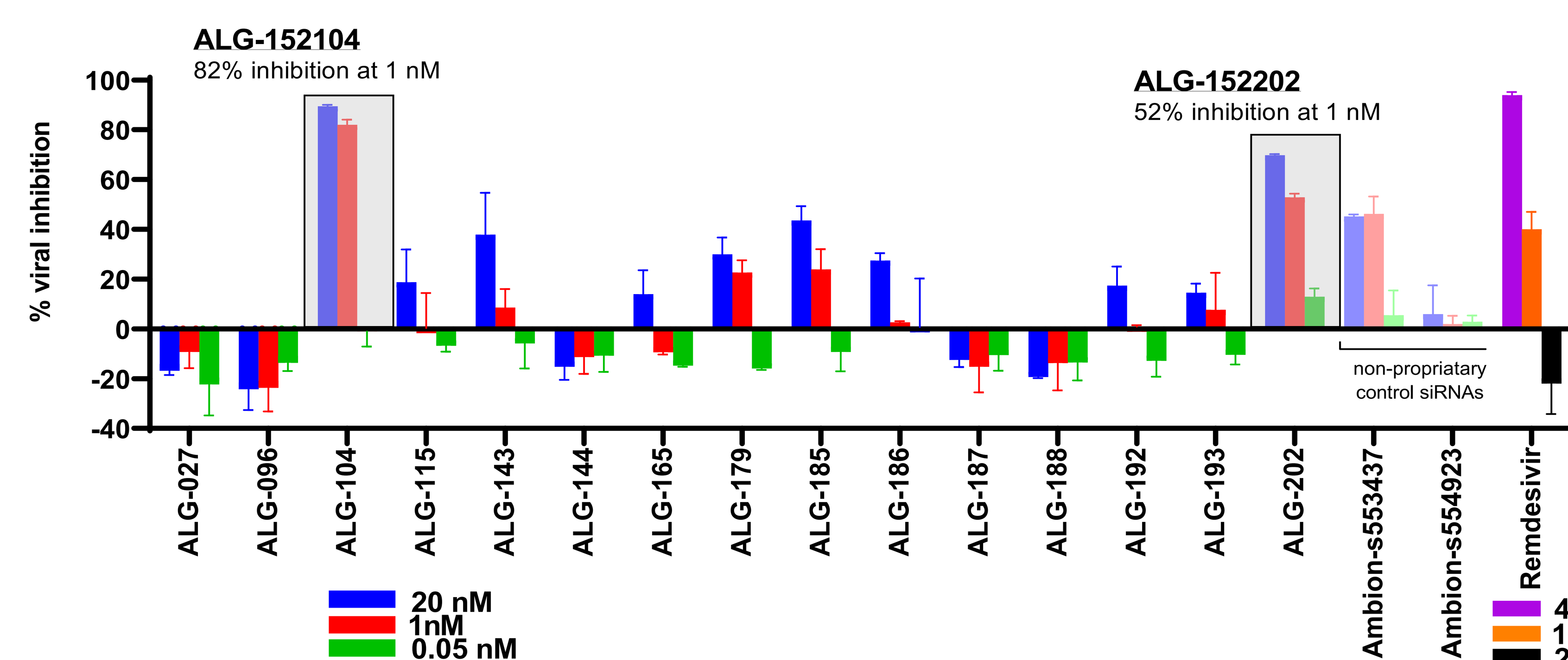
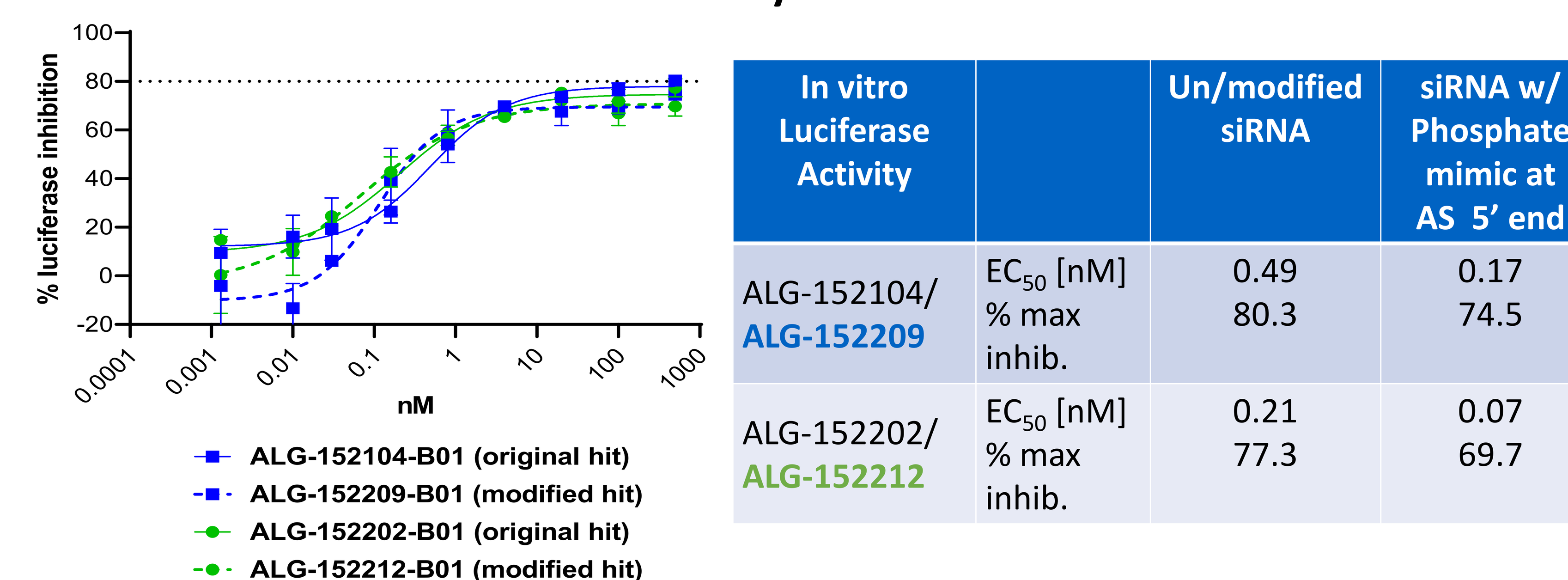


Figure 2: Antiviral activity of two potent siRNAs (highlighted) in the SARS-CoV-2 hACE-2 A549 assay where human ACE-2 expressing A549 lung epithelial cells were infected with nano-luciferase tagged SARS-CoV-2 followed by transfection of siRNAs at three concentrations for 48 hrs.

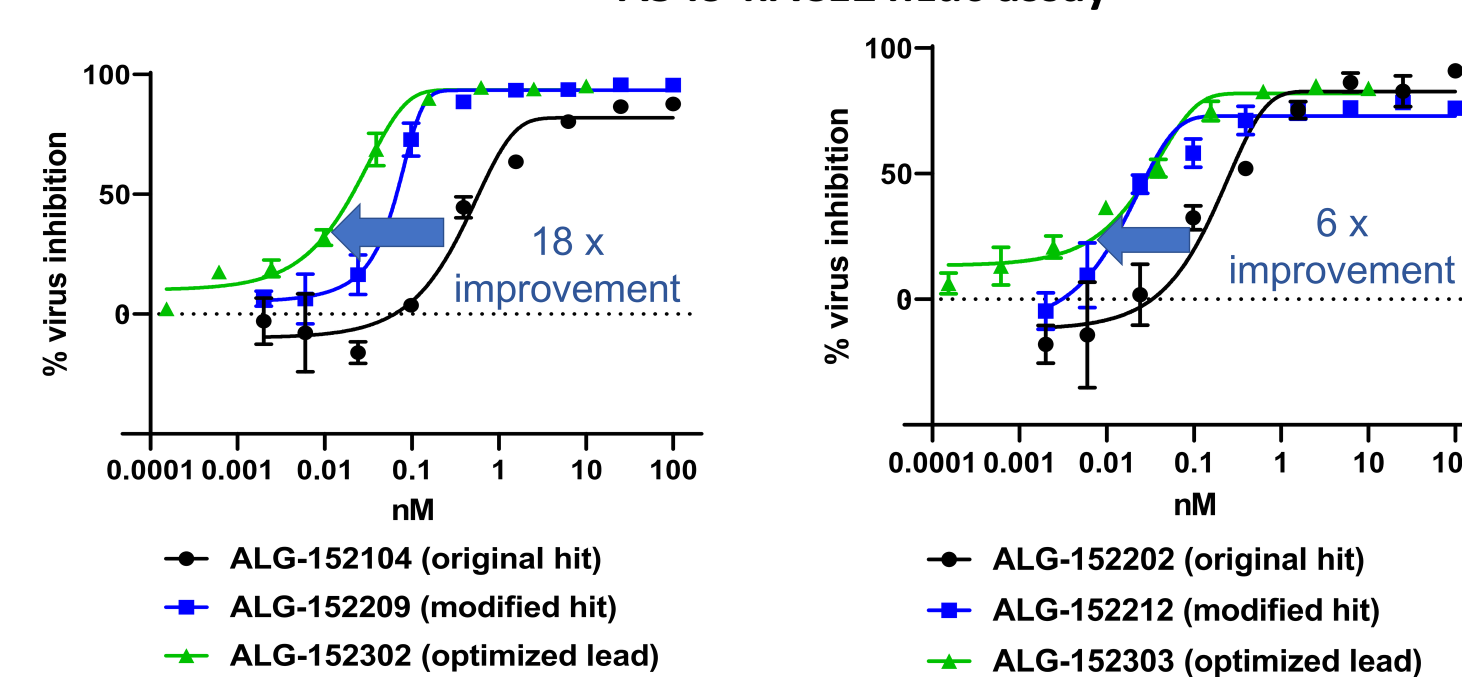
Luciferase assay in COS-7 cells



In vitro Luciferase Activity		Un/modified siRNA	siRNA w/ Phosphate mimic at AS 5′ end
ALG-152104/ ALG-152209	EC_{50} [nM]	0.49	0.17
	% max inhib.	80.3	74.5
ALG-152202/ ALG-152212	EC_{50} [nM]	0.21	0.07
	% max inhib.	77.3	69.7

Figure 3 and Table 1: psiCHECK-2 luciferase assay in Cos7 cells displaying addition of a phosphate mimic (dotted lines) to the 5′ end of the antisense strand, improved the potency of leads by 3-9-fold. Predictive of SARS-CoV-2/A549 assay to assess antiviral activity.

A549-hACE2 nLuc assay



Design	ALG #	EC_{50} [nM]	Max. Inhib. [%]	ALG #	EC_{50} [nM]	Max. Inhib. [%]
Original hit	152104	0.378	87.6	152202	0.122	90.0
Modified hit	152209	0.059	95.6	152212	0.011	76.1
Optimized lead	152302	0.021	95.6	152303	0.021	86.9

Figure 4 and Table 2: Antiviral activity of original siRNA screening hits (black), modified hits (blue) and the optimized lead siRNAs (green) in A549-hACE-2 cells, transfected with increasing concentrations of the siRNAs and infected with a nano-luciferase tagged SARS-CoV-2 virus. 24-48 hrs after infection, nano-luciferase activity was measured as a surrogate marker for viral infection and EC_{50} was determined using GraphPad Prism.

A549 - Cytotoxicity assay

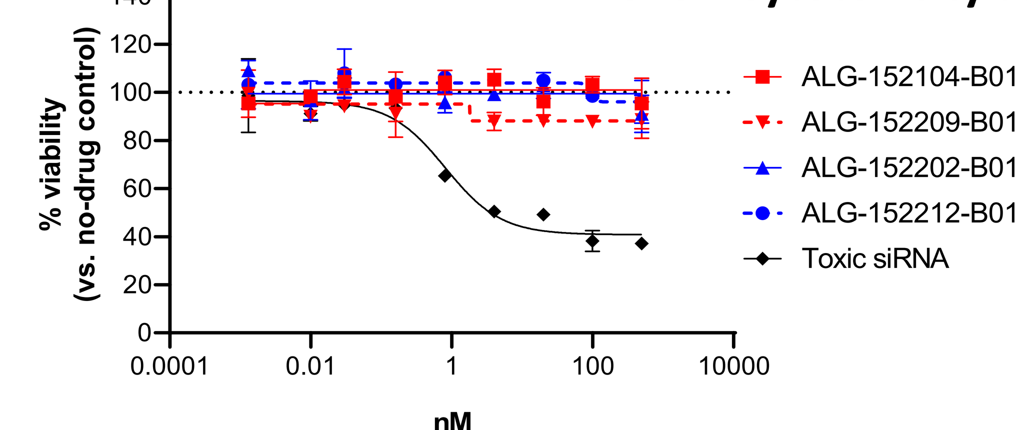
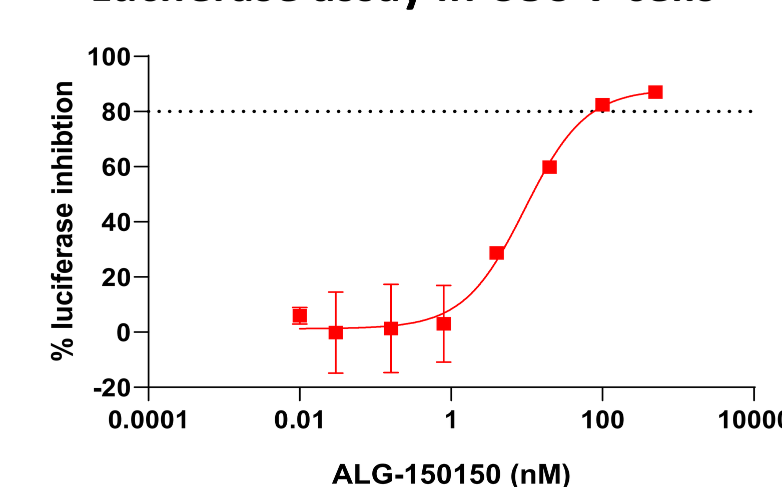


Figure 5: No cytotoxicity with original hits (bold lines) and lead siRNAs (dotted lines), transfected with increasing concentrations on A549 cells and tested 48 hrs after treatment via CellTiter-Glo®.

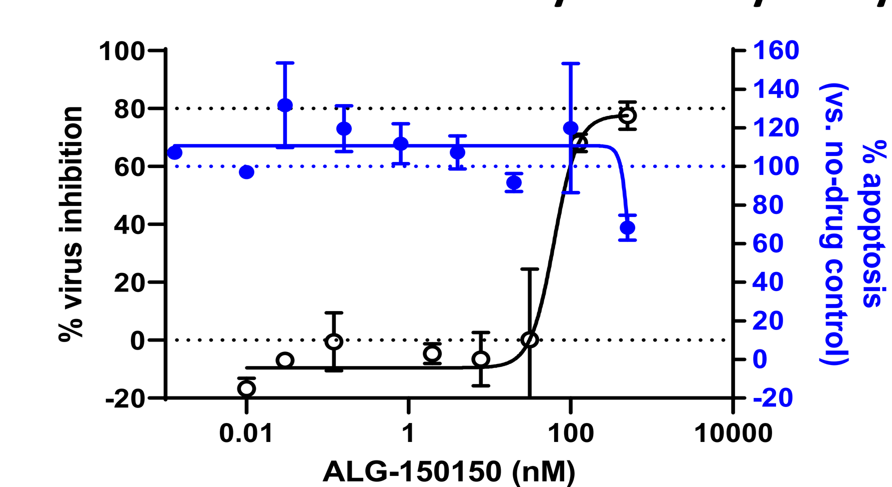
2. In vitro activity of ASOs

We identified 4 ASO hits from the initial screening using the psiCHECK-2 luciferase reporter assay, displaying EC_{50} values < 10 nM and maximum inhibition of reporter expression greater than 70%. We identified and optimized a lead ASO (ALG-150150) which displayed strong antiviral activity in the SARS-CoV-2 viral infection assay with EC_{50} of 62 nM, 78% max. inhibition. ALG-150150 did not induce cytotoxicity as measured by CellTiter-Glo® and did not activate caspase-3 at concentrations up to 500 nM in COS-7 and A549 cells.

Luciferase assay in COS-7 cells



A549-hACE2 nLuc and cytotoxicity assay



ALG-150150	Luciferase activity	Antiviral activity
EC_{50} (nM)	9.4	62.32
% max inhib.	88.2	77.5

Figure 6 and Table 3: Luciferase and antiviral activity of the lead ASO was tested in COS-7 and A549-hACE-2 cells respectively where Renilla luciferase and nLuc tagged SARS-CoV-2 activity was measured and EC_{50} was determined using GraphPad Prism.

3. In vitro off-target selectivity

To assess off-target effects, 10 different housekeeping genes were screened to assess endogenous expression via qPCR. Lead compounds do not show off-target RNA inhibition after 48 hr treatment in A549 cells. No cytotoxicity was observed at the concentrations tested. A follow-up RNAseq profiling in A549 cells is planned.

Gene	Pathway / Function
18S rRNA: 18S ribosomal RNA	Protein synthesis
ACTB: Beta-actin	Cell structure
TFG: Trafficking From ER To Golgi Regulator	Vesicle transport from ER to Golgi
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase	Glycolytic dehydrogenase
SFRS4: Serine and arginine rich splicing factor 4	mRNA processing
YWHAZ: 14-3-3 Protein Zeta/Delta	Adapter protein – cell survival, metabolism, etc.
HPRT1: Hypoxanthine phosphoribosyltransferase 1	purine salvage pathway
GUSB: Beta-glucuronidase	Glycosaminoglycan degradation
B2M: Beta-2 microglobulin	MHC class I – self antigen presentation
HMBS: Hydroxymethylbilane Synthase	heme biosynthetic pathway

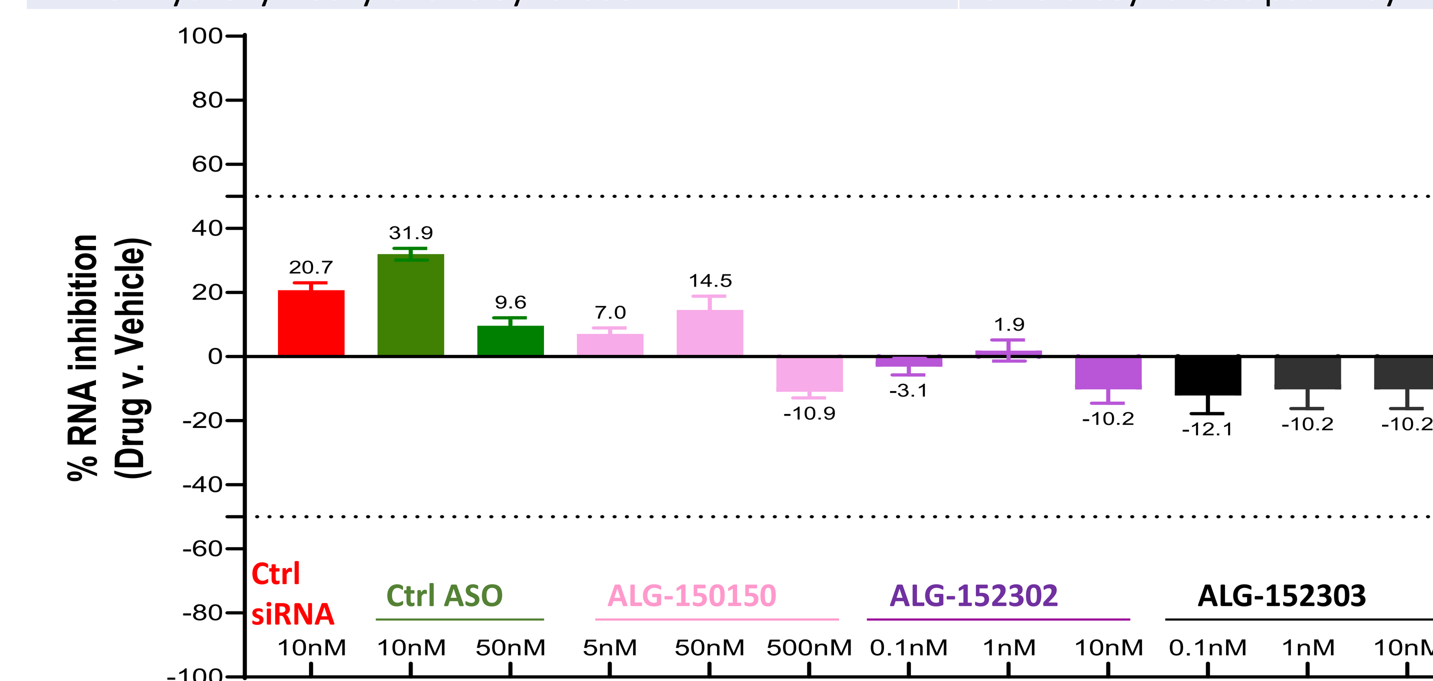


Figure 7 and Table 4: Both lead ASO and lead siRNAs were transfected at various concentrations along with appropriate controls in A549 cells for 48 hrs. 10 different housekeeping genes were assessed for endogenous expression via qPCR. All reference genes were averaged and ΔCt was calculated relative to Mock and % inhibition was calculated from Mock samples.

Conclusions

We developed a platform for designing effective siRNAs and ASOs against SARS-CoV-2 and identified highly potent oligonucleotides that can be used for therapeutic and preventative treatment of COVID-19. Current work focuses on optimizing the delivery of the lead compounds to bronchial epithelial cells.

References

[1] Connelly CM et al *J Biomol Screen.* 2012;17(6):822-828., psiCHECK-2 (Promega, Madison, WI) [2] Xie et al 2020 *Nat Commun* **11**, 5214 (2020)

Financial disclosures

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