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REVIEW ARTICLE

siRNA as a Potential Therapy for COVID-19

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Abstract: Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV2) is a highly contagious virus causing COVID-19 disease that severely impacted the world health, education, and economy systems in 2020. The numbers of infection cases and reported deaths are still increasing with no specific treatment identified yet to halt this pandemic. Currently, several proposed treatments are under preclinical and clinical investigations now, alongside the race to vaccinate as many individuals as possible. The genome of SARS-CoV2 shares a similar gene organization as other viruses in the Coronaviridae family. It is a positive-sense, single-stranded RNA. This feature suggests that RNA interference (RNAi) is an attractive prophylactic and therapeutic option for the control of this pandemic and other possible future pandemics of the corona viruses. RNAi utilizes the use of siRNA molecules, which are 21-29 nt duplexes RNA molecules that intervene with targeted gene expression in the cytoplasm by a specific mechanism of complementary destruction of mRNA. Previous experience with SARS-CoV and the Middle East respiratory syndrome (MERS) showed that siRNA molecules were effective against these viruses *in vitro* and *in vivo*. Moreover, there have been extensive advances in siRNA technology in the past decade from chemistry and target selection considerations; which concluded with the successful approval of two commercial products based on siRNA technology. In addition, the current knowledge of the genome structure and functionality of the corona viruses enables the recognition of conserved sequences to optimize siRNA targeting and avoid viral escape through mutations, either for the current SARS-CoV2 as well as future corona viruses.

Keywords: SARS-CoV2, Corona viruses, COVID-19, RNA interference, siRNA, respiratory.

1. INTRODUCTION

Severe Acute Respiratory Syndrome Coronavirus 2 (hereinafter referred to as SARS-CoV2) terrified the world's population and challenged the worldwide healthcare systems after the emergence of this new strain in late 2019 in Wuhan, China, leading to the unpredicted worldwide outbreak of the novel coronavirus disease, COVID-19 [1]. SARS-CoV2 is part of the Coronavirinae subfamily within the family Coronaviridae, which belongs to the Nidovirales order [2]. Members of the Coronavirinae subfamily are commonly referred to as coronaviruses, which are relatively large viruses that comprise a single-strand positive-sense RNA genetic molecule enveloped within a viral membrane characterized by surface projections. These projections are spike glycoprotein that make the studded viral envelope looks like a crown, giving the viruses their name [3]. The Coronavirinae subfamily (CoV) can be further divided into the alpha (α), beta (β), gamma (γ), and delta (δ) coronaviruses. Of interest are the beta coronaviruses class that includes seven viruses that are known to infect humans. The first four are HCoV-OC43, HCoV-HKU1, HCoV-229E, and HCoV-NL63, which usually cause self-limiting upper respiratory infections in adults. However, severe and life-threatening lower respiratory tract infections were also reported in immunocompromised patients or susceptible populations, such as infants and elderlies [2, 4, 5]. The other three viruses are the severe acute respiratory syndrome virus (SARS-CoV), which emerged in 2002, the Middle East respiratory syndrome virus (MERS-CoV), which emerged in 2012, and the current SARS-CoV2 [6]. SARS-CoV2 attacks the lower respiratory system of humans and is more transmissible and contagious than the other subclasses of the beta coronaviruses [7]. The pathogenicity and contagiousity of the beta coronaviruses depend on several structural and nonstructural proteins, expressed by the emerged virus where many host proteins of the infected cells become hijacked by the viral protein to allow viral replication and spreading. Spike glycoprotein (S protein), nucleocapsid protein (N), nsp1, ORF3b encoding protein and ORF6 encoding protein are the main pro-

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teins found to participate in the pathogenicity and fatality of SARS-CoV2. The S protein, for example, mediates the host cell invasion by binding to angiotensin-converting enzyme 2 (ACE2) receptor on the surface membrane of host cells [8, 9]. Beta CoVs were found to share variable levels of genome similarity and basic structure. SARS-CoV2 shares 79-82% sequence identity with SARS-CoV and 50% sequence identity with MERS-CoV [10, 11]. Apparently, SARS-CoV2 is sufficiently different from other CoVs and it was considered a new human-infecting beta coronavirus. Therefore, the genome similarity is enough to anticipate that potential treatments for SARS, in particular, should be in principle helpful for developing COVID-19 therapeutics, as well as whatever new CoVs may emerge in the future [12]. Accordingly, the genome organization of SARS-CoV2, as well as its encoded proteins, are presented and compared thoroughly with those of SARS-CoV. Lessons learned from previous studies that established the potential of RNAi therapeutics against SARS-CoV are deliberated. Since COVID-19 is a respiratory disease, it was deemed necessary to review as well the pulmonary delivery of siRNA. Whatever experience that has been gathered for pulmonary delivery of siRNA should be also, in principle, applicable for siRNA targeting SARS-CoV2. This review proposes that interfering the viral protein expression may render SARS-CoV2 vulnerability and protect the host innate immunity. It may prove as a successful strategy to target SARS-CoV2 replication and spreading.

2. THE GENOME ORGANIZATION OF CORONAVIRUSES

The genome organization of SARS-CoV2 has been sequenced extensively by various laboratories across the world and compared to other beta CoVs [2, 10, 11, 13-15]. However, the roles or functionalities of various segments in the SARS-CoV2 genome are mostly based on analogy with other beta CoVs characterized previously. SARS-CoV2 has a positive-sense single-stranded RNA comprising 29.9 kb nucleotides encoding 9860 amino acids, in comparison to SARS-CoV and MERS-CoV whose genomes size is 27.9 kb and 30.1 kb, respectively [16, 17]. The RNA strand has a 5′ methylated cap and 3′ polyadenylated tail structure. It is considered one of the largest genomes among the RNA viruses. Several structural and non-structural proteins for which the SARS-CoV2 genome encodes have been identified. The structural proteins include spike glycoprotein (S) which consists of two domains, namely S1 and S2, envelope protein (E), membrane protein (M), and nucleocapsid protein (N), from 5′ to 3′ gene order. It also contains two flanking untranslated regions, the 5′- and 3′-UTRs, that are 265 and 358 nucleotides long, respectively [10].

SARS-CoV2 genome has two major open reading frames, namely ORF1a and ORF1b, that extend from base 266 to base 21563 representing ~ 71% of the virus genome [18]. In addition, there are another ten functional smaller ORFs including those encoding for the previously mentioned structural proteins. Within ORF1a and ORF1b lie the Replicase gene (Rep), which translates to two complex and large polyproteins: pp1a and pp1ab, which consist of 4,405 and 7,096 amino acids, respectively [19]. Once translated, these two polyprotiens are proteolytically processed by viral-encoded proteases including chymotrypsin-like protease (3CLpro) or main protease (Mpro) and papain-like protease into sixteen nonstructural proteins [2, 13]. They are numbered nsp1-nsp16 according to their order from the N- terminus to the C-terminus of the ORF 1 polyproteins. There are also several other nonstructural proteins that are encoded by intergenic regions located between S and envelope protein (E), between membrane protein (M), and nucleocapsid protein (N) or downstream of N. Some of these nonstructural proteins have unknown functions and vary widely between the subfamilies of coronaviruses. Interestingly, pp1a and pp1ab are translated directly from the genomic RNA. The other remaining proteins of the beta CoVs are translated from a nested set of 9 subgenomic mRNAs which are produced by a discontinuous transcription process [20].

3. SARS-COV2 *VERSUS* SARS-COV: GENOMIC COMPARISON

Phylogenetically, SARS-CoV2 has been found to share a sufficient level of sequence identity with the 2003 SARS--CoV (Table **1**). The 16 nonstructural proteins encoded by ORF1a and ORF1b appear to be significantly preserved with an identity of the amino acid sequences between the two viruses, varying between 68% and 100%. The leader protein, nsp1, is the first nonstructural protein of the coronaviruses' polyprotein. It promotes a selective host mRNA cleavage by an unknown mechanism after binding to the 40S ribosome of the host cell. In other words, it regulates the host and viral gene expression in favor of the viral gene translation and suppressing the expression of host genes including those involving IFN-dependent antiviral signaling pathways [21]. It is reported that nsp1 is highly divergent among CoVs and it is only encoded for by α and β CoVs. Nevertheless, the SARS viruses share 84.0 - 84.4 sequence identity as shown in Table **1**. This indicates similar biological functions despite the lack of overall sequence similarity.

The lowest identity and similarity (68% and 90%, respectively) were found for nsp2, which is expected as it is the most variable protein among the 16 nsps in coronaviruses [22]. Although the function of nsp2 is unknown, Angeletti and coworkers suggested that the stabilizing mutations in this nsp may play a role in the ability SARS-CoV2 displayed in being highly contagious in comparison to the previous related viruses [23]. Next is nsp3m which shares 76% identity. Nsp3 is a large multifunctional protein with up to 16 different domains and regions. Among the various functions of nsp3, it is essential to form the replication-transcription complex. The domain organization of nsp3 differs between various CoVs. Nevertheless, 8 domains and 2 transmembrane regions are usually conserved [24]. Taking this into account, a 76% identity may be considered as a significant level of similarity between the two viruses. All other nsps share ≥ 80 sequence identity, with 9 of the remaining 13 nsp (nsp 4 - nsp16) being more than 93% similar. Notable among these nsps are the RNA-dependent RNA

Table 1. Amino acid sequence identity and similarity between SARS-CoV-2 and SARS-CoV human viruses.

Protein	No. of Amino Acids		Chan *et al.*, 2020 [9]	Yoshimoto *et al.*, 2020 [17]	
	SARS-CoV-2	SARS-CoV	Sequence Identity	Sequence Identity	Sequence Similarity
NSP1	180	180	84	84.4	93.9
NSP2	638	638	68	68.3	90
NSP3	1945	1922	76	76	91.8
NSP4	500	500	80	80	95
NSP5	306	306	96	96.1	99.7
NSP6	290	290	88	88.2	98.3
NSP7	83	83	99	98.8	100
NSP8	198	198	97	97.5	100
NSP9	113	113	97	97.3	99.1
NSP10	139	139	97	97.1	99.3
NSP11	13	13	85	84.6	100
NSP12	932	932	96	96.4	99.4
NSP13	601	601	100	99.8	100
NSP14	527	527	95	95.1	99.1
NSP15	346	346	89	88.7	97.7
NSP16	298	298	93	93.3	99
Spike	1273	1255	76	76	91.5
ORF3a	275	274	72	72.4	90.2
ORF3b	22	153	32	Not reported	Not reported
Envelope	75	76	95	94.7	97.4
Membrane	222	221	91	90.5	98.2
ORF6	61	63	69	68.9	93.4
ORF7a	121	122	85	85.2	95.9
ORF7b	43	44	81	85.4	97.2
ORF8 *vs.* ORF8a	121	39	Not reported	31.7	70.7
ORF8 *vs.* ORF8b	121	84	40	40.5	66.7
Nucleoprotein	419	422	94	90.5	97.2
ORF10 *vs.* ORF9b	38	98	73	28.6	52.4

polymerase (nsp12), helicase (nsp13), and endoribonuclease (nsp15), which share 96%, 100%, and 89% identity between the two viruses, respectively. These three enzymes are responsible for viral replication and transcription [10, 14].

With regard to the remaining accessory proteins encoded by other ORFs, their functions and roles in the pathogenicity and contagiosity of SARS-CoV2 are not fully understood yet. Reports are emerging discussing possible roles in antagonizing some immune proteins or shutting down key parts of the human immune system to attenuate the host antiviral immune responses. ORF3a accessory protein is suggested to have a pro-apoptotic activity in different cell lines [25]. However, the pro-apoptotic mechanism is different and the activity is weaker for SARS-CoV2. This is in agreement with the less virulence of SARS-CoV2 compared to SARS--CoV. On the basis of amino acids sequence, the proteins share 72% sequence identity between the two viruses. Another accessory protein is encoded by ORF3b, which is one of the most abundant proteins that are dominantly expressed in patients during early phases of infection of SARS-CoV2 [26]. The amino acid sequence is only 32% identical with SARS-CoV2 and SARS-CoV. This is the least similarity percentage found upon comparison of the genomic structure of these two phylogenetically closely-related viruses. ORF3b length is different between the two viruses, with only 22 amino acids (66 bp) in SARS-CoV2. This is considerably shorter than the 153 amino acids of SARS--CoV ORF3b protein. The presence of four premature stop codons in ORF3b gene is behind truncation. This protein has the ability to inhibit interferon-1 production [27]. Nevertheless, the lack of C-terminal region (residues 115-154), which is believed to attenuate the anti-IFN-I activity in SARS--CoV2 ORF3b boosted the antagonistic activity against IFN-I of SARS-CoV2. Moreover, variants in ORF3b, due to the deletion of one of the stop codons, may result in the emergence of highly pathogenic SARS-CoV2 that expresses elongated ORF3b proteins with higher interferon-1 suppression activities [28].

ORF6 and ORF8 encoded proteins are other two accessory proteins that function to suppress primary interferon production and interferon signaling, thus suppressing the host innate immune activation [29, 30]. ORF6 shares a higher identity (69%) in amino acid sequence than ORF8 (40%) between the two viruses. ORF8 protein isolated from ear-

ly-phase patients infected with SARS-CoV in 2003 was found to hold a full length of 122 amino acids. Strikingly, a 29-nucleotide deletion was reported for virus isolates from mid- and late- phase human patients. This deletion resulted in a split of ORF8 into two ORFs, 8a and 8b, encoding 39- and 84- residue polypeptides, respectively [31]. Zhang and coworkers reported a potential immune evasion strategy of SARS-CoV2 through the ORF8 protein, in addition to its type I interferon antagonistic activity [32]. It involved the down-regulation of surface expression of MHC-I molecules after direct binding with the ORF8 protein, subsequently disrupting antigen presentation and reducing recognition and elimination of infected cells. Contrariwise, neither the intact ORF8, ORF8a, nor ORF8b proteins of SARS-CoV could execute such functionality. Apparently, the ORF8 protein of SARS-CoV2 is a novel protein that is distant in sequence and functionality from that of SARS-CoV.

The four main structural proteins (S, E, M, and N) are encoded by ORFs 10 and 11. The spike (S) glycoprotein of coronaviruses, in general, mediates the virus entrance into host cells. This protruding transmembrane protein is composed of two subunits: S1 and S2, which are non-covalently bound in the pre-fusion state prior to attachment to the host cells [33]. The distal S1 subunit contains the receptor-binding domain, and thus it is responsible for binding to ACE2 receptors in the case of SARS-CoVs. Once bound, the two subunits are cleaved by host proteases to activate the fusion of the viral and cellular membrane mediated by the S2 subunit [34]. Although the SARS-CoV2 encodes a longer spike protein than that of SARS-CoV, both viruses were found to share a 76% amino acid sequence identity in total. Specifically, the S1 subunit is ~ 70% similar, while the highly conserved S2 subunit is 99% similar for the two viruses. The S1 subunit has three segments: a signal peptide, followed by an N-terminal domain and receptor-binding domain. Most importantly, the receptor-binding domain of both viruses was found to be highly conserved with 99% similarity. The sequence conservation explains the structural similarity and comparable affinity to ACE2 receptors for the S glycoprotein of both viruses [11, 35]. On the other hand, the envelope protein (E), membrane protein (M), and nucleocapsid protein (N), share a higher identity than the spike protein, with 95%, 91%, and 94% of the amino acid sequence, respectively.

ORF7a encodes 121 amino acids type-I transmembrane membrane protein whose exact function remains unclear. Pro-apoptotic effects of ORF7a proteins were hypothesized and linked to several interactions between this protein and cellular proteins that were identified. It also appears to act as a suppressor of siRNA activity in mammalian cells. Subsequently, it protects the viral genome expression during the infection against the RNA-silencing mechanisms of the host [36]. ORF7b is a short (43 amino acids only) type III transmembrane protein that is restrictedly localized in the Golgi complex. It is also believed to induce apoptosis in transfected cells, but to a lesser extent than ORF7a [37]. Apart from sequence identification and comparison to SARS-CoV (~ 85% identity), these two proteins are perhaps the least investigated proteins in the novel SARS-CoV2 virus owing to their unclear functionalities and role during viral infection.

The last accessory protein is ORF10 protein. It is a 38 amino acid protein that is unique to SARS-CoV2. Its functionality has not been investigated yet. SARS-CoV, on the other hand, contains a 98 amino acid ORF9b protein which is not present in the new virus. Contradicting sequence identity comparisons are reported between the two proteins which should have not been compared since they are two distant proteins with unknown functionalities (Table **1**).

4. RNA INTERFERENCE

Gene therapy is a promising therapeutic strategy in medicine that aims to target a specific gene in specific cells of targeted tissues. Initially, the focus of gene therapy was toward introducing a gene through a plasmid DNA vector to replace a mutated gene such as in cystic fibrosis or to encode for a therapeutic protein to prevent or treat a disease [38]. Later, gene therapy was found to have more applications through reduction or preventing the production of a protein from its corresponding gene, referred to as gene silencing. The silencing effect can be achieved by intervening with the gene expression before translation. RNA interference (RNAi) is a natural biological mechanism for gene silencing in most eukaryotic cells. It promotes the degradation of mRNA in the cytoplasm through a complementary sequence-recognition mechanism prior to its translation [39, 40]. Since its discovery in 1998, RNAi has been established as a potential therapeutic approach for the treatment of several genetic, metabolic, infectious, and malignant diseases by selective knockdown of disease-causing genes. However, the prospective outcomes of RNAi can be only achieved if the gene silencing element is successfully delivered to the target cells cytoplasm, where the RNA-induced silencing complex (RISC) is located [41].

RNAi can be mediated by a variety of molecules including small interfering RNA (siRNA), short hairpin RNA (shRNA), and micro-RNA (miRNA) molecules. siRNAs are non-coding double-strand RNA molecules that are 19-30 nucleotides in length with two 3′ 2-nucleotide overhangs. They are negatively charged macromolecules with an average molecular weight of ~14 kDa [42]. Once in the cytoplasm, it associates with the inactive RISC. The two strands get separated releasing the sense (passenger) strand and leaving the antisense (guide) strand in the activated RISC to bind a complementary mRNA. The Argonaute endoribonuclease component of the activated RISC cleaves the mRNA, therefore blocking its translation into full functional proteins [43]. Identification of target sequences and the subsequent selection, validation and synthesis of complementary siRNA molecules can be performed successfully through a variety of tools [44].

5. GENOME OF SARS-CoV TARGETED BY siRNA

Unlike small drug molecules which require spatial recognition of a protein target conformation, siRNA molecules rely on Watson-Crick base pairing with mRNA to induce its si-

lencing function. This would of course shorten the tedious and lengthy drug discovery process required in the development of safe and effective small drug molecules. Moreover, any gene of interest can be, in theory, targeted by siRNA including the ones that do not have druggable active sites by the selection of the right complementary nucleotide sequence. Currently, there are two approved siRNA therapeutics: ONPATTRO® and GIVLAARI® for the treatment of the transthyretin amyloidosis (TTR-mediated amyloidosis, a hereditary form), and acute hepatic porphyria, respectively. Several other siRNA molecules for the treatment of a wide range of conditions are currently undergoing clinical testing in phases 1-3 [45]. Viral infections are generally difficult to treat, and some viruses do not have druggable targets. siRNA-mediated gene silencing has been investigated as a potential treatment for several viral infections. Examples include the Lassa Virus [46], Coronavirus NL63 [47], MERS-CoV [48], H5N1 influenza virus [49], Herpes simplex virus [50], Hepatitis C Virus [51], and Human immunodeficiency virus [52].

In order to establish RNAi as a therapeutic tool for SARS-CoV-2, potential RNAi targets within the virus genome must be identified first. Fortunately, several RNAi targets have been already identified for the phylogenetically related SARS-CoV, and their inhibitory siRNA or shRNA molecules have been shown to induce effective suppression of the virus in various biological models. Libraries of siRNA molecules were investigated against various targets of SARS-CoV genome with varying effectiveness. The most effective siRNA molecules reported so far are summarized in Tables **2** and **3**. These siRNA molecules were generated for the silencing studies by one of the following methods: A) expression in cells from an siRNA expression plasmid vector, B) chemical synthesis, or C) *in vitro* transcription from template deoxynucleotides.

Table 2. Effective siRNA molecules that were reported based on DNA targeted sequence.

siRNA	DNA-targeted Sequence (5' - 3')	Target Region (Nucleotide)	Target Protein	siRNA Generating Method	Delivery Platform	Validation Cell Line/Animal	References
pSR02	cttacatagctcgcgtctc	14450 to 14468	RNA polymerase	pSUPER.retro vector	Lipofectamine 2000	Vero E6 cells infected with HKU-39849 strain	[101]
pSR03	gaatattaggcgcaggctg	15877 to 15895	RNA polymerase				
Ei2	aaggagttcctgatcttctggt	206 to 227 of E sequence	Envelope	PCR based siRNA expression cassettes	FuGENE6®	NIH 3T3 cells transfected with envE-pcDNA3.1 or RDRP-pcDNA3.1 vectors	[63]
Ri3	aaggacatgacctaccgtagac	394 to 415 of NSP12 sequence	RNA polymerase				
-	ccaaccaacctcgatctc	NA	Leader	pBS/U6/L-RNAi vector	CalPhos®	293T and Vero E6 cells transfected with pEF-Bos/L-GFP or pDsRead-1.1/L-RFP	[102]
SC02	aagctcctaattacactcaac	21553-21573	Spike	Chemical synthesis	Lipofectamine 2000	FRhk-4 cells infected with HKU-66078 strain	[51]
SC05	aaggatgaggaaggcaattta	13530-13550	RNA polymerase				
SC14	aaggataagtcagctcaatgc	17544-17564	helicase				
SC15	aactggcacactacttgtcga	20843-20863	endoribonuclease				
RNAi1	gagacatatctaatgtgcc	1358-1376 from the first ATG of the cDNA of the gene	Spike (S1)	pBS/U6 vector	CalPhos®	293Tcells transfected with pCMV-Myc vector and Vero E6 cells infected with BJ01 strain	[103]
RNAi2	gggctaccaccttatgtcc	3081-3099 from the first ATG of the cDNA of the gene	Spike (S2)				
N388	ggcatcgtatgggttgcaact	388-407 of N sequence	Nucleocapsid	pMD-18T vector	Lipofectamine 2000	293T cells and BALB/c mice muscles transfected with pN-EGFP vector	[62]
si-M1	ggtgactggcgggattgcgattg	221-242 of M gene	Membrane	pBS/U6-siM1-3 vectors	ProFection®	HEK293T cells transfected with pCMV-Myc-M vector	[104]
si-M2	ggcgctgtgacattaaggacc	466-486 of M gene	Membrane				
Si-M3	aacgacaatattgctttgcta	637-657 of M gene	Membrane				

(Table 2) contd….

siRNA	DNA-targeted Sequence (5' - 3')	Target Region (Nucleotide)	Target Protein	siRNA Generating Method	Delivery Platform	Validation Cell Line/Animal	References
si-N213	ggcgttccaatcaacaccaa	213-233 of N gene	Nucleocapsid	Chemical synthesis of oligos which were subcloned into pB-S/U6 vectors	ProFection®	HEK293 transfected with pEGFP-Np or pCMV-Myc-Np vectors	[105]
si-N863	ggaccaagacctaatcagac	863-883 of N gene	Nucleocapsid				
si-N1240	gagcttctgctgattcaact	1240-1260 of N gene	Nucleocapsid				
siSARS-S2	gggctatcaacctatagat	NA	Spike	Chemical synthesis	Lipofectamine 2000	Vero E6 cells infected with the Hong Kong strain of SARS-CoV	[106]
iSARS-S3	caaggcgattagtcaaatt	NA	Spike				
siSARS3'UTR2	cgtaactaaacagcacaag	NA	3'-UTR region				
siRNA 2	aataaacatgttcgtttagag	NA	sgRNA 2 (spike)	pRNAT-U6.1/Hygro vector	Lipofectamine 2000	Vero E6 cells transfected with pXJ-CS-7a, pXJ-CS-8a-HA or pXJ-CSn-sp1-HA vectors and Vero E6 cells infected with SARS CoV	[54]
siRNA 3	aaatccataagttcgtttaga	NA	sgRNA 3 (3a/3b protiens				
siRNA 7	tctctaaacgaacatgaaa	NA	sgRNA 7 (7a/7b proteins)				
shRNA-A	atcttaggattgcctacgc	NA	RNA polymerase	pSilence1.0-U6 vector	Lipofectamine 2000	HeLa and 293 cells transfected with pIRES-2-EGFP/RDRP or pCMV-HA-RDRP vectors and Vero E6 cells infected with SARS CoV-p9	[107]
siRNA-1	gatggagagccttgttctt	262-280 of nsp1 gene	Leader	pSilencer 3.1-H1 vector	Electroporation, Lipofectamine 2000	Vero E6 cells infected with BJ01 strain	[61]
siRNA-2	cagccctatgtgttcatta	445-463 of nsp1 gene	Leader				
siRNA-3	ctcactcgtgagctcaatg	766-784 of nsp1 gene	Leader				

Table 3. Effective synthetic siRNA molecules.

siRNA	siRNA Anti-sense Strand (5' - 3')	Target Region (Nucleotide)	Target Protein	Delivery Platform	Validation Cell Line/Animal	References
siRNA-5	agaagaucaggaacuccuucaTT	NA	Envelope	Lipofectamine 2000	Vero E6 cells transfected with pCDNA3.1/E, pCDNA3.1/M, or pCDNA3.1/N vectors	[64]
siRNA-6	guuccaggaguguuuaagcuTT	NA	Membrane			
siRNA-16	guuugauugggguccauuaucTT	NA	Nucleocapsid			
siSC2	guugaguguaauuaggagcTT	21553-21573	Spike	intranasal instillation of siRNA solution in D5W	Rhesus macaque infected with PUMC-01 strain	[60]
siSC5	uaaauugccuuccucauccTT	13530-13550	RNA polymerase			
SARSi-S	cacugauuccguucgagauc	23150-23169	Spike	OligoFectamine®	FRhk-4 cells infected with GZ50 strain	[108, 109]
SARSi-E	cguuucggaagaaacagguac	26113-26133	Envelope			
SARSi-N	caagccucuucucgcuccuc	28648-28667	Nucleocapsid			
SARSi-M1	ugcuugcugcugucuacag	26576-26594	Membrane			
SARSi-M2	guggcuuagcuacuucguug	26652-26671	Membrane			
SARSi-2	guacccucuugauugcaucTT	NA	Replicase	OligoFectamine®	FRhk-4 cells infected with GZ50, GZ34, HKR1, andHKR2 strain	[110]
SARSi-3	gagucgaagagaggugucuTT	NA				
SARSi-4	gcacuugucuaccuugaugTT	NA				
siSARS1	uaaauugccuuccucauccTT	13547-13567	RNA polymerase	Lipofectamine 2000	Vero E6 cells infected with Frankfurt 1 isolate	[53, 111]
siSARS3	aauuaccggguuugacaguTT	14595-14615	RNA polymerase			
S-siRNA1	gagcuuugagauugacaaauu	403-423 of spike gene	Spike	Lipofectamine 2000	HEK293T cells transfected with pEGFP-S vector	[112]
S-siRNA2	cccuuucuuugcuguuucuuu	871-891 of spike gene	Spike			

From the plethora of studies reported so far, it can be noticed that targets covering the whole SARS-CoV genome, even including the 5′-UTR and the 3′-UTR regions, were screened for suppression of the virus by RNAi. Among these, the spike protein and RNA polymerase are the most targeted, followed by the other three structural envelope, membrane, and nucleocapsid portions. Other parts of the genome were also targeted such as the leader protein (nsp1), helicase (nsp13), endoribonuclease (nsp16), 3a/3b proteins, and 7a/7b proteins. It should be recognized that the effective siRNA molecules and their targets (summarized in Tables **2** and **3**) had been discovered following a tedious screening of a large number of carefully selected nucleotide targets all over the viral genome by different researchers. For example, the number of chemically synthesized siRNA duplexes was 48 by Zheng *et al.* [53], 26 by Shi *et al.* [54], and 34 by Elmen *et al.* [55]. Out of these 108 siRNA molecules, only 9 were found to induce significant RNAi activity.

It should be emphasized that the effectiveness of RNAi-based therapeutics is not through a selective knockdown of a specific viral gene. Instead, it is mediated via the destruction of the viral mRNA, as evident by the reduction of viral genome copy number. Therefore, Zheng *et al.* [53] recommended that this can be achieved if ORF1a and ORF1b, representing the first two-thirds of the CoV genome, are targeted instead of the other ORFs in the right-hand third region of the viral genome. This conclusion was reached upon screening 32 siRNA molecules targeting ORF2-ORF9, where only one molecule resulted in moderate activity as compared to the weak inhibition elicited by the others. That is also supported by the findings of Akerström *et al.* [56] who designed an siRNA molecule to target sgRNA 7, encoding for 7a/7b accessory proteins. It has been shown that these two proteins are not necessary for the replication of the virus as the recombinant virus lacking 7a and 7b can replicate as efficiently as the wild-type virus [57]. Accordingly, the significant inhibition of virus production in Vero E6 cells stably-infected with SARC-CoV is the direct result of viral mRNA destruction.

6. LESSONS LEARNED

Studies exploring RNAi as a potential therapeutic approach for coronaviruses have been reviewed in the past [58, 59], as well as recently [60]. To re-summarize their findings is not the purpose of this review but to highlight certain observations that are believed to pave the way towards developing a successful therapy for the COVID-19 outbreak. Out of the 20 studies summarized in Tables **2** and **3**, 17 were reported between 2003 and 2007; just after the SARC-CoV 2003 outbreak. Huge advances have been accomplished since then with regard to the chemical modification of siRNA molecules to render them more resistant to RNases and phosphatases [61, 62]. The improved biological stability is directly related to a more favorable pharmacokinetic behavior [63, 64]. In addition to the enhanced activity, certain chemical modifications were found to efficiently suppress immunostimulatory siRNA-driven innate immune activation [65]. Others may help in reducing off-target-induced toxicity [66, 67]. Apparently, the current siRNA development and synthesis technologies offer much-enhanced potency and reduced toxicity [45]. Moreover, it allows for high throughput screening of siRNA molecules and targets to identify lead molecules. In the studies reported in Table **2**, nine of them utilized a plasmid vector to generate siRNA molecules inside the cells. The limitation of such technology for high throughput screening is obvious. Moreover, it necessitates the use of an effective vector to aid the delivery of the plasmid into the nucleus of the cells.

The delivery platforms used in that previous reports are also summarized in Tables **2** and **3**. Apparently, lipofectamine 2000 was the delivery system of choice used by various groups. FuGENE6® and OligoFectamine® are other lipid-based systems for gene delivery. On the other hand, physical methods for gene delivery were also used, such as electroporation or CalPhos® and ProFection®. The last two are transfection reagents based on calcium phosphate and calcium chloride, respectively. Again, vectors for gene delivery, whether viral or non-viral (chemical and physical), are a continuously developing field since the 1990s. More efficient and less toxic delivery platforms are still being developed [68]. The formation of a stable, safe, and effective delivery vector, from a pharmaceutical perspective, is a prerequisite for the development of an appropriate dosage form for this kind of therapy. For screening purposes in tumorous cells, the use of any effective-proven delivery platform is justified regardless of its toxicity or practicality for in vivo use.

In addition to delivery vectors, bioconjugation of siRNA to molecules from natural origin, mostly by a cleavable linkage, is another promising strategy for the delivery and targeting of siRNA. There are several siRNA conjugates that have been reported in the literature. Examples include conjugation to lipophilic molecules, aptamers, ligands, antibodies, peptides, or polymers [69]. This will allow for targeting of siRNA through specific binding of the biogenic molecule to receptors on the cell membrane or facilitating the penetration of the cell by natural transport mechanisms [61]. N-acetylgalactosamine as a ligand for siRNA delivery represents the most successful example so far as it was used in the currently approved GIVLAARI®. It interacts with the asialoglycoprotein receptor that is highly expressed on hepatocytes and thus, allows the siRNA therapeutic molecule to target them for the treatment of acute hepatic porphyria [70].

We know so far that SARS-CoVs infect cells that express angiotensin-converting enzyme 2 (ACE2) through binding of the spike protein to this receptor to initiate receptor-mediated internalization [71]. The use of siRNA bioconjugates that target these cells could enhance the potency, minimize side effects and off-target effects, and prolong the half-life of siRNA molecules. Especially in the early phase, when the infection is still localized in the respiratory system before spreading to other organs [72].

As mentioned earlier, there are two approved siRNA therapeutics. Since these two products have undergone rigorous development process to successful marketing, it is worth paying attention to the delivery approach used for each. The

first one is ONPATTRO®, which is a 2 mg/ml concentrate for solution for infusion. It contains Patisiran as an active ingredient formulated as lipid nanoparticles (LNP) (ONPATTRO® SPC). These LNP are ≤ 100 nm in size with a low surface charge. They are also characterized by their high encapsulation efficiency and can be produced by a scalable manufacturing process and possess adequate product stability. The lipids used for preparing the LNP are DLin-MC3-DMA, PEG_{2000}-C-DMG, DSPC, and cholesterol. DLin-MC3-DMA is an ionizable aminolipid with a pKa of 6.5. This value is very close to the optimum value of 6.4 which has been shown to be very critical, as a deviation of 0.5 from this pKa value resulted in a significant loss of activity [73]. On the other hand, the incorporation of the pegylated lipid at the right proportion with DLin-MC3-DMA resulted in the formation of 20-100 nm nanoparticles [74]. Moreover, the use of a myristoyl derivative of the pegylated lipid was crucial to ensure that it would be exchanged out of the LNP with lipoprotein particles in the plasma in order to allow the interaction of the LNP with the target cells [75].

GIVLAARI®, on the other hand, is a solution for subcutaneous injection that contains 200 mg Givosiran sodium corresponding to 189 mg Givosiran per ml. Givosiran is a double-stranded synthetic chemically modified siRNA, which is conjugated to a triantennary N-acetyl galactosamine ligand to facilitate delivery of the siRNA to the liver. The formulation of this product is much simpler than the ONPATTRO® as the active molecule is dissolved in water for injection and adjusted to pH 6.5-7.5 with sodium hydroxide or phosphoric acid (EMA public assessment report for GIVLAARI®). These two approved products are both injectable products and not intended for pulmonary delivery. The delivery was based on two different approaches as one was formulated as LNP and the other is a simple aqueous solution of a bioconjugated siRNA. Yet, both are proven effective. Although pulmonary delivery of siRNA had its own limitation as discussed later, it was anticipated to be much easier and straightforward than the parenteral route of administration.

Interestingly, Li, *et al.* [76] used an siRNA combination as a prophylactic as well as a treatment in rhesus macaques infected with SARS-CoV. The siRNA was delivered as is in a solution form in D5W by intranasal instillation. Analysis of oropharyngeal swab samples of rhesus macaques after 4 days of SARS infection by quantitative real-time PCR revealed that 75% of the samples treated with an siSC-2-siSC5 combination (prophylactic, co-delivery or post-exposure) did not have any detectable SARS-CoV RNA. Contrastingly, the viral RNA was 100% detectable in all of the untreated animals and those treated with nonspecific siRNA. This was also accompanied by a much lower number of infected lung cells in the treatment groups indicating that the siRNA induced effective inhibition of SARS-CoV replication and spread within the monkey lungs. Obviously, the siRNA molecules were effective without the use of delivery vectors. So, the lack of pharmaceutically approved delivery vectors should not hamper the clinical development of RNAi-based therapies against corona viruses.

siRNA molecules have been shown to be effective prophylactically and therapeutically. The pre-existence of siRNA molecules within the cells inhibited SARS-CoV infection and replication *in vitro* [53, 77]. This indicated that the viral genomic mRNAs are sensitive to the pre-existing siRNA within host cells. This effect was confirmed *in vivo* using a Rhesus macaque model and the body temperature of the infected monkeys as a marker for the severity of SARS-like symptoms [76]. The group treated prophylactically had a significantly lower body temperature (38.7 °C vs 38.5 °C for control) than groups given siRNA treatment concomitantly with the viral infectious load or as a post-exposure dose. In a comparable relative work, Zhao *et al.* [78] constructed a pU6-shN388 plasmid vector expressing the effective N388 siRNA molecule against the N protein of SARS-CoV. They co-injected it with N-EGFP expression plasmid encoding the N and EGFP proteins to murine muscles. This resulted in a reduction of the expression of both proteins to ~ 20% for 16 days post-injection.

Among the two tasks, using siRNA prophylactically appears to be the easier one. Once the host is infected and the virus replication is activated, a tremendous load of viral mRNA would be generated, which may exceed the silencing capacity of RNAi machinery in the cells. What performs well as a prophylactic does not necessarily have the same effectiveness therapeutically. For example, among the 48 siRNA molecules that were screened by Zheng and coworkers [53], only four siRNA molecules, SC02, SC05, SC14, and SC15 targeting spike, RNA polymerase, helicase, and endoribonuclease, respectively, showed promising effectiveness in preventing SARS-CoV infection in FRhK-4 cells. However, when the same dosage of siRNA used in the prophylactic study was applied post-transfection, only SC15 was able to induce > 70 inhibition activity as measured by a relative reduction in viral genome copy numbers. It is noteworthy that the most effective siRNA molecule prophylactically, which is SC15, was also the most effective therapeutically.

In order to improve the silencing outcomes, the dose may be increased or a combination of siRNA, preferably targeting different regions in the virus genome, can be used. With regard to the first option, the RNAi effect against the expression of RNA polymerase from an RDRP-pcDNA3.1 plasmid that was co-transfected with varying concentrations of shRNA was found to follow a dose-dependent effect [79]. The same was observed for siRNA molecules targeting the E, M, and N genes co-transfected with a previous plasmid vector encoding these genes in the Vero E6 cell line [80]. In an opposing study, tripling the dosage of a moderately active siRNA has been shown to have no significant improvement in prophylactic efficacy [53]. Such contradicting reports emphasize the need for dosage optimization of effective molecules, which is a prerequisite for the development of efficient RNAi-based therapeutics. As mentioned earlier, Li and coworkers delivered 30 µg of siSC2-siSC5 combination in 3 ml of D5W solution to Rhesus macaques by intranasal instillation. The used dose can be approximated to 8-9.7 µg/ kg; depending on the average weight of the animals. This study offers an initial dosing guidance for future

preclinical and clinical studies. However, it should be kept in mind that dose optimization is very likely to be required especially if a carrier was used to aid the delivery of siRNA molecules.

On the other hand, siRNA combinations have been demonstrated to promote significantly higher anti-SARS therapeutic effect *in vitro* and *in vivo* [53, 76]. Moreover, it is logical to anticipate that the use of siRNA combinations will diminish the risk of the virus escaping RNAi through mutations in its genome.

7. siRNA DELIVERY TO LUNG: CHALLENGES AND OPPORTUNITIES

Delivery of siRNA therapeutics to the lungs, by systemic or pulmonary administration, has been documented as a promising approach for the treatment of respiratory disorders [81]. Compared to systemic administration, the pulmonary route is non-invasive and well accepted by patients. It offers direct delivery of therapeutics to the airways for the treatment of local conditions, leading to the need for lower doses and minimizing the systemic side effects [82, 83]. In addition, the pulmonary route offers avoidance of siRNA molecules whole body-distribution and rapid clearance by serum nuclease in the blood [84-86].

Nevertheless, pulmonary drug delivery, in general, is exposed to many external and internal barriers. The local pulmonary delivery of siRNA therapeutics to the lungs faces many barriers involving the physiological factors that can be further classified into extracellular and intracellular barriers. Extracellular barriers include airways defenses, such as cough clearance, high branching structure, mucociliary clearance as well as mucus and alveoli defenses, such as alveolar fluid, alveolar macrophage, enzymatic degradation and pulmonary surfactants [86-89]. Intracellular barriers for siRNA delivery to lung cells include cellular uptake efficiency and escape from endosomes to reach the cytoplasm where they can exert their therapeutic activity [86].

The branched structure of the respiratory airways represents the primary barrier in pulmonary drug delivery [90]. To exert the therapeutic effect, the therapeutic particles should deposit in the lower airways. Inhaled particle deposition in the lungs occurs by different mechanisms including inertial impaction, diffusion, gravitational sedimentation, interception and electrostatic precipitation. The optimal particle size for lung deposition is between 1 and 5 μm [91, 92]. Large particles (> 6 μm) are exposed to be impacted and trapped on the upper airway wall, while small particles (ranging from 0.1 to 1 μm) are easily exhaled during breathing. Smaller particles (under 100 nm) are successfully deposited in the alveolar space by increasing diffusional mobility [93].

siRNA molecules, whether naked or as particulates, should overcome the extracellular barriers and reach the target cells, then they need to overcome the intracellular barriers. siRNA molecules need to reach the cytoplasm, where the RISC locates, and the gene silencing process happens. Cellular uptake of siRNA molecules involves various endocytic mechanisms [94-97]; this includes clathrin-dependent endocytosis, which is the most common route of cellular entry for macromolecules [98]. Following cell entry, siRNA molecules are entrapped in the endosomes where acidification occurs. Then, the endosomes fuse with the lysosomes, which contain hydrolases that degrade the siRNA molecules [98, 99]. Therefore, siRNA molecules should escape from endosomes at an early stage to exert their silencing effect [100, 101].

siRNA macromolecules are negatively charged hydrophilic molecules that are susceptible to nuclease degradation. They are incapable to cross the biological membrane on their own and reach the site of action at an effective concentration. Yet, several proof-of-concept studies have shown that naked siRNA molecules can elicit considerable activity when delivered as such without the use of a carrier [81]. However, a delivery vector would indeed aid siRNA molecules to efficiently escape the aforementioned barriers through the enhancement of cellular uptake as well as protection of siRNA molecules to increase their biological half-life [85, 102, 103]. The vector should have many characteristics including condensing siRNA into nanosized particles, protecting siRNA from enzymatic degradation, enhancing cellular uptake, promoting endosomal escape and releasing its cargo into the cytoplasm, besides having negligible toxicity [104-106].

The efficacy of siRNA to treat various lung diseases via intratracheal and/or intranasal routes for siRNA delivery to the lung has been investigated *in vivo*. A measurable efficacy with significant improvement of the signs and biomarkers has been reported. For lung cancer treatment, inhalable aerosols of siRNA directed against the sodium-dependent phosphate co-transporter 2b (NPT2b) were developed. The siRNA was entrapped in a poly (amino ester) carrier and administered twice a week for four weeks to mice with lung cancer. The results showed that lung cancer growth, cancer cell proliferation and angiogenesis were suppressed, and apoptosis was facilitated [107]. In another study, lipid-based nanoparticles of siRNA were administered by intranasal inhalation to mice with orthotopic lung cancer. In comparison to intravenous treatment, the pulmonary-delivered siRNA showed an enhancement in antitumor activity and reduction of adverse effects [108].

Regarding respiratory viral infection treatment, 100 μg of naked nucleocapsid-targeting siRNA in phosphate-buffered saline was administered intranasally to mice before infection (via a single dose) or after infection (via multiple doses) with respiratory syncytial virus. A significant reduction of viral load was achieved in both prophylactic and therapeutic regimens [109]. Also, anti-GFP-949 siRNA and anti-nucleoprotein (NP)-1496 siRNA chitosan nanoparticles were administered to mice with the Influenza virus. The results showed protection of 50% of mice against a lethal challenge of mouse-adapted influenza viruses [110].

Another wide-spread pulmonary condition is asthma. For asthma treatment, siRNA molecules silencing the suppressors of cytokine signaling (SOCS) proteins, which are

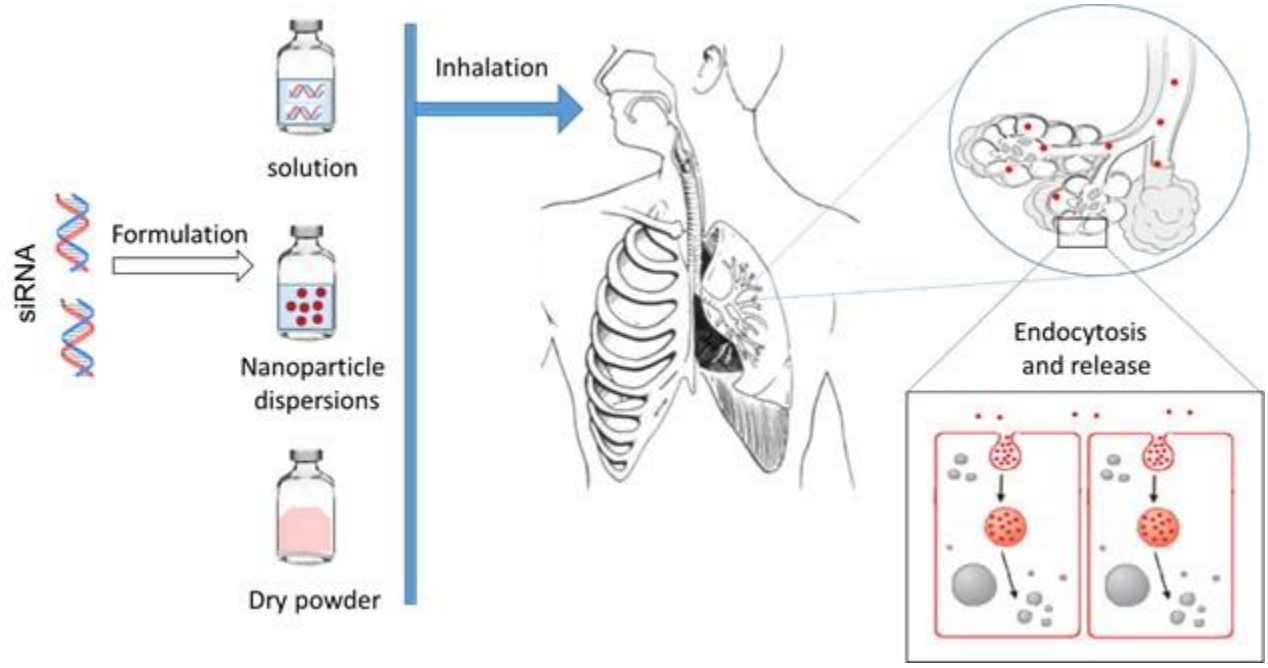

Fig. (1). Schematic representation of siRNA delivery to the lungs: from formulation to release into the cytoplasm of alveolar cells. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

increased in asthmatic conditions, were administered intranasally to the lungs of mice with chronic asthma. The SOC-S3- naked siRNA led to an improvement in the eosinophil count and mucus secretion and a reduction in lung collagen [111]. Another anti-Interleukin-4 and anti-Respiratory Synaptic Virus naked siRNA molecules were given intranasally to OVA-sensitized mice with RSV-induced exacerbation. The results showed inhibition of pathological signs of asthma (airway inflammation and hyperresponsiveness), reduction in eosinophilia and neutrophilia in the lung, suppression expression of IL-4 associated with allergic response and inhibition of RSV replication [112]. Moreover, naked siRNA molecule targeting STAT6, a major driver of bronchial inflammation, was administered intranasally to OVA-sensitized rats for 3 consecutive days. The treatment showed a significant reduction in allergen-induced lung inflammation [113].

Finally, the siRNA approach was also investigated as a potential treatment for cystic fibrosis. Intranasal anti-periostin naked siRNA was administered. Tomaru *et al.* [114] showed that the siRNA therapy reduced lung collagen deposition and decreased expression of profibrotic cytokines and decreased bronchoalveolar lavage fluid and lung tissue levels of total TGF-1 and lung tissue TGF-1 mRNA expression in mice.

Generally, the major challenges for pulmonary delivery of siRNA include poor correlation between *in vitro* and *in vivo* studies, hard translation of the information from animal models to humans because of the differences in anatomy and physiology of the respiratory tract between animal and human and the administration routes used in animal studies are not suitable for human use, such as the intratracheal route. Moreover, the difficulty in evaluation of the delivery efficiency of the formulation before entering the clinical study. As discussed above, several proofs of concept studies have successfully established the therapeutic benefits of siRNA treatment for many lung diseases in animal models. Still, the most crucial challenge that must be overcome now is the development of inhalable stable siRNA formulations for human use in the laboratory in order to use them for lung diseases in clinical trials [85, 93, 106].

CONCLUSION

The continuing rise in infection cases and deaths associated with COVID-19 pandemic has been accompanied by unprecedented race at two fronts: first for the development of an effective vaccine against SARS-CoV-2, and the second focused on the discovery of effective treatment options. In the latter capacity, the efficacy of several existing antiviral drugs is being clinically evaluated for the treatment of COVID-19. Examples of antivirals that are undergoing drug repurposing include Remdesivir, Favipiravir, Umifenovir, and Lopinavir/Ritonavir [115]. Unfortunately, no strong clinical evidence has been provided to support their full clinical usefulness in terms of efficacy and safety against SARS-CoV-2 [116]. On the 22nd of October 2020, the United States FDA approved the use of Remdesivir for the treatment of COVID-19 requiring hospitalization. This approval was based on its beneficial outcomes with regard to time-to-recovery clinical endpoint. Remdesivir was statistically superior to placebo in reducing the median time for recovery in mild, moderate and severe COVID-19 cases. Despite this ac-

complishment, the difference in mortality between the Remdesivir group and the placebo group was, unfortunately, statistically insignificant (www.accessdata.fda.gov).

The fact that SARS-CoV2 genome is a positive-sense single-stranded RNA makes RNAi an attractive therapeutic option for COVID-19. Especially if an inhalation siRNA is developed so it can be administered through the same route the virus uses to cause the infection. Vir Biotechnology and Alnylam Pharmaceuticals are collaborating to screen 350 siRNAs targeting various regions of SARS-CoV2 genome *in vitro* to identify lead candidates. The companies announced that potential candidates will undergo preclinical evaluation by the end of the year. The success of these studies and hopefully, the future human clinical studies and authorization of the therapy, would represent a game-changer in the field of respiratory disorders. Of course, treating COVID-19 is the priority now, but this would also offer insurance against future pandemics from other coronaviruses that might emerge. Principally, if the conserved region of the genome among various members of the Coronaviridae family is targeted, we should learn Lessons from our previous mistake with SARS--CoV in 2003 when we thought that there was no need to invest in finding a cure since the epidemic was controlled. Had our thinking been different back then, we might not have had to endure the heavy consequences we are suffering now from the SARS-CoV2. Moreover, the successful development of RNAi-based inhalation therapeutic against COVID-19 that can reach the alveoli and lung parenchyma (Fig. **1**) would pave the road for treating other respiratory disorders. In addition to infections, lung cancers, asthma, and idiopathic pulmonary fibrosis could be the next targets for RNAi-based inhalation therapeutics.

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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