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Coronavirus RNA proofreading: molecular basis and therapeutic targeting

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Dear Editor,

I am hereby electronically submitting our manuscript titled "Coronavirus RNA proofreading: molecular basis and therapeutic targeting" for your consideration for publication in *Pharmacological Research*.

Existing review articles focus on general therapeutic approaches targeting coronaviruses (CoVs) (Zumla et al., 2016; Ghosh et al., 2020; Li & De Clercq, 2020). Our review specifically focuses on targeting the proofreading mechanism of CoVs that is critical for antiviral drug resistance. To date, our manuscript is the first review that discusses both chemical (e.g. nucleoside-based inhibitors) and genomic (e.g. CRISPR) approaches as potential anti-CoV therapeutics.

We expect that our review will be of great interest to medicinal chemists, pharmacologists, virologists and chemical biologists who read your journal and will lead to subsequent follow-up development of novel anti-CoV therapeutics by other research groups.

I assure you that the manuscript is not under consideration for publication elsewhere. I hope that you will find the manuscript suitable for publication in *Pharmacological Research*, and I very much appreciate a quick peer-reviewing decision due to the time-sensitive nature of this topic.

Yours sincerely,

Billy

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Coronavirus RNA proofreading: molecular basis and therapeutic targeting

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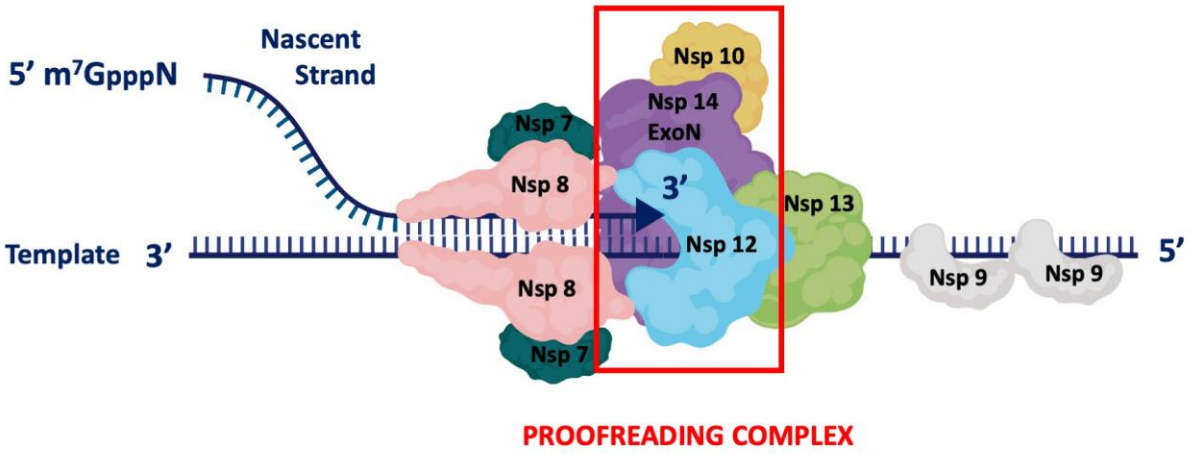
Highlights

- Molecular basis of CoV proofreading machinery and design of novel anti-SARS-CoV drugs
- Repurposing nucleoside analogues to combine with other therapies against SARS-CoV-2
- Highly specific nucleic acid-based techniques as a potential antiviral strategy

Abstract

The coronavirus disease 2019 (COVID-19) that is wreaking havoc on global public health and economies has heightened awareness about the lack of effective antiviral treatments or vaccines for human coronaviruses (CoVs). Many current antivirals, notably nucleoside analogues, exert their effect by incorporation into viral genomes and subsequent disruption of viral replication and fidelity. The development of anti-CoV drugs has long been hindered by the capacity of CoVs to proofread and remove mismatched nucleotides during genome replication and transcription. Here, we review the molecular basis of the CoV proofreading complex and evaluate its potential as a drug target. We also consider existing nucleoside analogues and novel genomic techniques as potential anti-CoV therapeutics that could be used individually or in combination to target the proofreading mechanism.

Graphical abstract



Keywords

COVID-19; Exonuclease (ExoN); non-structural protein 14 (nsp14); SARS-CoV-2; Coronavirus (CoV); ExoN proofreading

1. Introduction

Coronaviruses (CoVs) are a group of related viruses that belong to the *Coronaviridae* family in the order *Nidovirales* [1]. They cause respiratory tract and gastrointestinal tract diseases of varying severity in mammals including humans, and birds (reviewed in [2]; [3]). They are enveloped, single-stranded positive-sense RNA viruses with some of the largest known viral genomes, ranging from approximately 26kb to 32kb [4]. The surface of each virion is decorated with characteristic club-shaped glycoprotein spikes that bind to host receptors and confer specificity and infectivity [5]. These spikes, when the spherical virion particles are viewed under an electron microscope, resemble the solar corona from which they get their name [6].

The current 2019/2020 pandemic is caused by SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2), also known as COVID-19 (coronavirus disease 2019). SARS-CoV-2 is one strain of the severe acute respiratory syndrome-related coronavirus species (SARSr-CoV) in the genus *Betacoronavirus* [7]. The SARSr-CoV strains include SARS-CoV (now called SARS-CoV-1) that was responsible for the SARS outbreak in 2002 and numerous other strains that cause diseases in bats and certain other mammals [8]. Another related species in the *Betacoronavirus* genus (MERS-CoV) caused the outbreak of Middle East respiratory syndrome disease in 2012.

The bulk of current knowledge of coronavirus molecular biology is based on data obtained studying members of the *Betacoronavirus* genus including the well-characterised animal CoV model, murine hepatitis virus (MHV) and those that have caused the aforementioned lethal zoonotic infections in the 21st Century: MERS-CoV and SARSr-CoV strains. Studies of the latter since their emergence in 2003 have generated a vast body of literature, and this, along with growing data on the new strain, SARS-CoV-2, will therefore be the major focus of this review.

2. The coronavirus genome

2.1. Genome organisation

The polycistronic RNA genome of SARSr-CoV is approximately 30kb and encodes 14 open reading frames, some of which overlap [9]. It is capped at the 5' end, has a 3' polyA tail, short 5' and 3' UTR sequences that form regulatory stem-loop structures and a single leader sequence proximal to the 5' end (**Figure 1**). A transcriptional regulatory sequence (TRS) precedes most ORFs. The 5' proximal end of the genome contains two large open reading frames: ORF1a and ORF1b, that together encode the replicase polyprotein and comprise approximately two thirds of the genome. These ORFs are translated from the viral RNA genome to produce two polyproteins: pp1a and, as a result of a programmed -1 ribosomal frameshift, the C-terminally-extended pp1ab. They are cleaved into 16 non-structural proteins (nsps) by ORF1a-encoded proteases nsp5 (chymotrypsin-like protease, $3CL^{pro}$, also called main or M^{pro}) and papain-like protease (PL^{pro}) residing in subunit nsp3. In addition to protease functions, nsps are involved in modulating the host cell environment, anchoring the viral replication complexes to subcellular domains and driving genome replication, transcription and mRNA processing. Nsp1 is involved in blocking the host innate immune response by inducing cellular mRNA degradation and preventing host RNA translation [10]. Nsp2 may play a role in disrupting intracellular host signalling [11]. Nsp4 is a putative transmembrane scaffold protein that is involved in the formation of double membrane vesicles (DMVs); the location of the viral replicase complex in infected cells [12,13]. nsp6 has been implicated in the generation of autophagosomes for the degradation of immunomodulatory proteins [14]. Nsps 4-16 are conserved across all identified CoVs and through experimental evidence or structural and homology-based analyses are predicted to be crucial for RNA synthesis and modification [15]. Nsp7 and nsp8 form a hexadecameric complex harboring presumed processivity function to the replication complex [16]; Nsp8 is a primase that synthesises short oligonucleotides that are then extended by the RNA-dependent RNA polymerase (RdRP) [17]; Nsp9 is a single-stranded RNA binding protein [18]; Nsp10 acts as a cofactor of nsp14 and nsp16 and promotes exoribonuclease (ExoN) and 2'-O-methyltransferase (2'-O-MT) activity, respectively [19]; Nsp12 functions as an RNA-dependent RNA Polymerase (RdRP) [20]; Nsp13 acts as a dual-function RNA helicase-ATPase [21]; Nsp14 has both exoribonuclease (ExoN) and methyltransferase (N7-MTase) activity [22]; Nsp15 is an endoribonuclease [23]; and nsp16 is an RNA 2'-O-methyltransferase

[24]. The function of the small polypeptide nsp11 is currently unknown [25]. The remaining one third of the genome contains the viral structural protein genes common to all CoVs (spike, envelope, membrane and nucleocapsid) and several ORFs encoding accessory proteins that vary in number between different CoVs. SARSr-CoV strains contain 8 (SARS-CoV-1) and 6 (SARS-CoV-2) accessory genes respectively with most variation between the two strains found in ORFs 3 and 8.

2.2. Genome replication and transcription

In common with other (+) strand RNA viruses, replication of the CoV genome involves continuous negative strand RNA synthesis to create a full-length complementary template and the subsequent copying of this template into multiple positive strand progeny genomes. These reactions are mediated by the viral RNA-dependent RNA polymerase (RdRP), the non-structural protein nsp12 encoded by ORF1b. There is evidence that both 5′- and 3′- end RNA elements are required for the production of progeny positive-strand RNA from the intermediate negative-strand RNA, suggesting that interactions between the 5′ and 3′ ends of the genome contribute to replication [26]. Viral replication is initiated when the RdRP protein binds to the 3’ end of the genome to initiate negative strand synthesis; a process stimulated by 3’ end RNA secondary structures and sequences [27]. In a process unique to RNA viruses in the order *Nidovirales*, transcription of the 3’ proximal structural and accessory protein genes is a discontinuous process that results in the production of a nested set of subgenomic mRNAs (sgmRNAs) that are 5’ and 3’ coterminal with the viral genome. CoV sgmRNAs have the single leader sequence from the 5’ end of the viral genome fused to the 5’ end of each mRNA sequence as a result of a discontinuous step involving template-switching during minus-strand synthesis. Transcription-regulating sequences (TRSs) located at the 3′ end of the leader sequence (TRS-L; “leader”) and preceding each viral gene (TRS-B; “body”) have core sequences in common that likely base-pair during transcription and promote leader-body joining [28,29]. The presence of a leader sequence in each transcript protects the CoV viral mRNA molecules from cleavage by the nsp1 protein that promotes degradation of host mRNAs, thereby suppressing the antiviral defence mechanisms [30]. **Figure 2** shows a summary of the life-cycle of the virus from initial recognition and binding to the host cell receptors, to eventual virion release.

2.3. The replication-transcription complex and mRNA processing

Coronavirus replication and transcription occurs in the cytoplasm of infected cells and is mediated by the replication transcription complex (RTC). This complex associates with modified endomembranes derived from the host endoplasmic reticulum that is transformed into viral replication organelles [31]. Anchorage of RTC to the membranes is mediated by the transmembrane domain of nsp3 together with transmembrane proteins, nsp4 and nsp6 [32]. The RTC comprises the majority of the nsps, the

nucleocapsid protein and a multitude of host proteins. The viral proteins considered to be the "core" RTC are the RNA-dependent RNA polymerase (RdRp; nsp12), the nsp13 helicase and processivity factors nsp7 and nsp8 [29] (**Figure 3**). The coronavirus nsp13 is a helicase that can unwind dsRNA in a 5' to 3' direction with the resulting ssRNAs probably serving as templates for RNA synthesis by the RdRP. The helicase activity is increased by a direct protein-protein interaction with nsp12 suggesting that the efficiency of viral RNA synthesis is enhanced when these two proteins interact in a functional RTC [21,33].

The COVID-19 pandemic has accelerated research into the structural and functional features of coronavirus RdRP which is the main target of antiviral nucleoside inhibitors. The nsp12 protein has at least two functional domains. At the N-terminus is a nidovirus RdRP-associated nucleotidyltransferase (NiRAN) domain unique to nidoviruses [34]. The C-terminus possesses the canonical RdRP domain that contains the conserved motifs and structural features of other RNA polymerases including the "palm, fingers and thumb" subdomains of a "cupped right hand". The RdRP subdomains bind template RNAs and select and bind an appropriate NTP by formation of phosphodiester bonds, hence extending the 3' end of the nascent RNA chain with the incoming nucleotide [35]. Recently, several articles have reported 3D structures of the novel SARS-CoV-2 RdRP protein, alone or complexed with partner proteins, RNA substrates and inhibitor compounds [36–41]. *In silico* molecular modeling studies using the published sequence of SARS-CoV-2 RdRP and available solved structures of SARS-CoV-1 [39,40] and *in vitro* cryo-EM of SARS-CoV-2 RdRP [37,38] sought to map promising antiviral compounds including remdesivir and its active metabolite to the active site. Coronavirus RdRPs interact with the hexadecameric cofactor nsp7/nsp8 protein complex that confers processivity to the polymerase [42]. Cryo-EM studies of the SARS-CoV-2 RdRP in complex with nsp7 and nsp8 have revealed several conserved and novel structural features. Gao *et al.* reported the structure of the nsp12-nsp8-nsp7 complex and identified a novel β-hairpin at the N-terminus of RdRP in addition to conserved architecture at the polymerase core [36]. A subsequent study of the SARS-CoV-2 structure in its replicating form bound to nsp7-nsp8 and a minimal RNA substrate, revealed a refined structure of the RdRP-RNA complex [41]. According to the detailed structural analysis, RdRP complexed with nsp7 and nsp8 connects with over two turns of duplex RNA. Conserved residues in the nsp12 active site cleft bind the first turn of RNA and mediate polymerase activity. Two copies of the characteristically "golf-club" shaped nsp8 protein position the RNA duplex as it exits the cleft and long α-helix extensions in nsp8 project out along the existing RNA duplex and interact with bases far from the polymerase core [16]. These protrusions are predicted to form positively-charged "sliding poles" that could prevent premature dissociation of the replication machinery from its substrate. As well as providing insight into the processivity of the RdRP that is necessary for replication of the very long genomes of viruses in the *Nidovales* order, this report also modeled the binding of incoming nucleotides to the NTP site. This demonstrated that contacts between nsp12 and canonical NTPs are

conserved and, crucially, that the nucleoside analogue remdesivir triphosphate could also be accommodated in the NTP binding site due to a space that would house its additional 1'-CN moiety. The data presented in these recent studies will enable further detailed analysis of the mechanisms used by antivirals to inhibit the coronavirus RdRP.

After replication and transcription, CoV genomic and sgmRNA transcripts are capped at their 5' end and polyadenylated at their 3' end by processes similar to those found in eukaryotic cells [43,44]. Capping and methylating viral mRNAs protects them from degradation by cellular 5' to 3' exonucleases, facilitates recognition by the host translation initiation machinery and promotes escape from host antiviral responses that recognise uncapped RNAs as "non-self" [45,46]. Unlike cellular mRNA capping that takes place in the nucleus, viral capping occurs in the cytoplasm and consequently is mediated by viral proteins. [47]. Coronavirus mRNA cap structures consist of a 7-methylguanosine linked to the first nucleotide of the RNA transcript through a 5′–5′ triphosphate bridge (^{7m}GpppN), a structure known as a "cap-0''. The addition of a methyl group to the first RNA molecule at its 2'O-position, converts the structure into a "cap-1" (^{7m}Gppp$_{m2}$N) [29,45,47,48]. The capping pathway in coronaviruses comprises four sequential enzymatic reactions: 1) hydrolysis of the 5'-ɣ-phosphate of the nascent RNA chain by the nsp13 helicase protein RTPase activity; 2) addition of GMP to the 5′-diphosphate RNA by an as-yet unidentified guanylyltransferase (GTase) forming a 5'–5' triphosphate bond; 3) methylation of the N7-position of the guanylate cap by nsp14 N7-MTase, forming the "cap-0" structure; 4) methylation of the 2'O-position of the first RNA molecule in the chain by the nsp10/nsp16 2'O-MTase to form the "cap-1" structure [45,46]. The capped RNA molecules are recognised by the host eukaryotic initiation factor 4E (eIF4E) that subsequently recruits the cellular translational machinery [49].

The coronavirus replication-transcription complex that directs viral RNA synthesis and processing described above presents a number of logical targets for the development of improved antiviral strategies.

2.4. Nucleoside and nucleotide analogue inhibitors that target coronavirus genomes

Nucleoside and nucleotide analogue inhibitors (NIs) are chemically-synthesised and structurally altered purines and pyrimidines, that have a long history of use as anticancer, antiviral, antibacterial and immunomodulator drugs [50]. Since the first successful clinical use in the 1960s of an NI, 5-iododeoxyuridine, for the treatment of herpes virus infections, NIs are now the largest class of the small molecule group of antiviral therapeutics [51]. They are designed to mimic naturally-occurring nucleosides(tides) and be recognised as such by the viral and cellular transcriptional machinery.

However, once incorporated into RNA or DNA strands, foreign nucleotides can mispair with natural nucleotides during subsequent replication/transcription leading to the accumulation of mutations and loss of viral viability. Alternatively, their incorporation may prematurely terminate the growing RNA or DNA chains and disrupt subsequent replication and transcription. In addition, the pool of naturally-occuring cellular nucleotides may be depleted or biased by the presence of NIs [52].

While there are now many examples of successful clinical therapies using NIs to target a range of human and domesticated animal viruses [50], targeting the CoV replication and transcriptional machinery using NIs is challenging.

3. Coronavirus proofreading mechanism

RNA virus replication typically has a high error rate that results in the virus existing as diverse populations of genome mutants or "quasispecies" with varying degrees of fitness. This factor in itself challenges antiviral development against CoVs and other RNA viruses as they can rapidly develop resistance to drugs while maintaining viral genome fitness [53]. An additional barrier to the development of NIs in particular as antivirals against CoVs is the unique 3' to 5' exonuclease (ExoN) proofreading function in CoV nsp14 enzymes that confers a 20-fold increase in replication fidelity compared with other RNA viruses [54,55]. CoV genomes and others in the *Nidovirales* order are the largest and most complex known RNA virus genomes and nsp14 is highly conserved within the *coronaviridae* family [56]. The exonuclease proofreading function of nsp14 may have been a crucial factor in the expansion and maintenance of such large genomes to ensure replication competence.

3.1. Structural and functional basis of nsp14

The nsp14 exoribonuclease activity resides in the N-terminus of this bifunctional protein while the C-terminal end hosts the N7-methyltransferase activity that is crucial for mRNA cap processing [9,15,57]. The ExoN domain is proposed to correct errors made by the RdRP by removing mismatched nucleotides from the 3' end of the growing RNA strand [58]. The ExoN domain of the protein was first identified by comparative sequence analysis that revealed a distant relationship of the N-terminal part of the protein with the DEDD protein superfamily of cellular exonucleases [9,59]. DEDD refers to four conserved catalytic Aspartic acid (D)/Glutamic acid (E) (Asp/Glu) amino acids present in most members of the superfamily and required by the protein to form two metal binding sites that drive nucleotide excision by a two-metal-ion assisted process [60–62].

Biochemical evidence that nsp14 has exonuclease activity and could degrade ssRNA in a 3' to 5' direction was demonstrated in *E. coli* using recombinant SARS-CoV-1 nsp14 [22,61]. The activity was shown to require Mg^{2+} as a cofactor and that secondary structure of the RNA substrate was important.

Mutating the conserved D/E residues further demonstrated that the ExoN domain in particular has an important catalytic function [22]. While murine hepatitis virus (MHV) and SARS-CoV-1 virus ExoN mutants could replicate in cell cultures, albeit with growth defects, strikingly, the mutants exhibited mutator phenotypes with increased mutation densities and rates compared to WT nsp14, suggesting that the protein acts to mitigate the relatively low replication fidelity of RdRP [54,55]. Genetically-engineered ExoN mutants were also found to be sensitive to RNA mutagens such as ribavirin and 5-fluorouracil, and incorporated more mutations in the presence of these compounds [63].

Interactions between viral proteins play crucial roles in many processes during the viral infection cycle and a yeast two-hybrid screen of intraviral protein-protein interactions revealed nsp10 as an nsp14 interacting partner [64]. *In vitro* experiments in *E. coli* established that the preferred substrate of the nsp10-bound nsp14 is dsRNA with a 3' mismatch that mimics an erroneous replication product. While nsp14 has exonuclease activity alone, this study also demonstrated a >35-fold increase in nsp14 activity when bound to nsp10 and suggested a role for nsp10 in stabilising the ExoN active site in the correct conformation for substrate catalysis [19,65].

X-ray crystallography of the nsp14/nsp10 complex and its functional ligands was able to establish a number of crucial factors that highlighted distinct differences between nsp14 and its homologues in the DEDD superfamily and cast light on several features of the protein's structure and function [56]. The crystal structures were compared to known structures of DEDD family members; *E. coli* DNA polymerase I Klenow fragment and the ε subunit of polymerase III, and this revealed that the catalytic residues in nsp14 are Asp90, Glu92, Glu191, His268, and Asp273; the conserved Asp424 of the Klenow fragment and Asp103 of the ε subunit are replaced in nsp14 with Glu191. This makes nsp14 a "DEED outlier". In addition, a conserved Histidine residue upstream of the final Asp that places it in the DEDDh subfamily. Simultaneous mutations D90A and E92A impaired the exonuclease activity significantly, and mutations in E191A, H268A and D243A attenuated RNA degradation suggesting the importance of these amino acids in nucleotide excision. The residue previously assumed to be at the catalytic core, Asp243, is nevertheless highly conserved and mutating it abolishes nsp14 activity, though its role has yet to be deciphered.

In addition to the revised catalytic motif, this study revealed significant differences between nsp14 ExoN and other members of the DEDD exonuclease superfamily. While the core structural elements are similar (the protein is a twisted β-sheet composed of five β-strands and flanked by two α-helices), there are several striking differences; nsp14 ExoN has in addition two zinc fingers that are crucial for exonuclease activity and two regions that interact with nsp10. Zinc finger 2 lies in proximity to the catalytic core and disruption of the domain via C261A or H264R mutations abolished enzyme activity suggesting that it has a role in catalysis. Zinc finger 1 mutations, on the other hand, resulted in

insoluble nsp14 proteins when expressed in *E. coli* and this, along with its structure suggested a role in the structural stability of nsp14.

The five catalytic DEEDh residues are predicted to coordinate two Mg^{2+} ions that assist the removal of misincorporated nucleotides. MgA would activate a water molecule to initiate a nucleophilic attack on the phosphate of the substrate nucleotide and MgB would then facilitate the exit of the product. Chen et al. demonstrated a two-metal binding mode for divalent cations by nsp14 using isothermal titration calorimetry. However, in the crystallography study only one Mg^{2+} was observed, coordinated by Asp90 and Glu191. This could be explained by lack of substrate or product binding [56,61].

The two regions that interact with cofactor nsp10 encompass amino acids 1-76, a long, flexible region, and 119-145, a β-hairpin structure containing β5 and β6. The complexed structure revealed that there is a one-to-one ratio of nsp10 to nsp14 molecules. Mutations in the nsp10 domain that interacts with nsp14 resulted in a decrease in viral replication fidelity and the structure of the complexed proteins suggested that nsp10 confers structural integrity and stability to the ExoN domain of nsp14 [56].

A study using pull-down and active enzyme assays to identify interacting partners confirmed that the nsp12/nsp7/nsp8 complex can interact with nsp14 with no apparent loss of polymerase processivity or nsp14 exonuclease/N7-guanine cap methyltransferase enzymatic activities. Adding nsp10 to the complex during exonuclease assays increased the ExoN activity suggesting that nsp10 can either enhance ExoN activity *in situ* in the active complex, or partially displace nsp14 from the nsp12/nsp7/nsp8 complex to activate ExoN separately [42]. Another study that used crystallography, small-angle X-ray scattering (SAXS) methods and mutagenesis of key residues found that nsp12-RdRP interacts with both the ExoN and N7-MTase domains of nsp14. Additionally, this study showed that nsp14 has a flexible hinge region separating the ExoN and N7-MTase domains that facilitates substantial conformational changes. This hinge may act as a molecular switch allowing different functions and interactions with diverse protein partners and RNA substrates. These data together demonstrate the integration of RNA polymerization, proofreading, and cap-modifying activities into a flexible multifunctional protein complex [66] **(Figure 3)**.

A recent report demonstrated that the proofreading exoribonuclease ExoN domain in nsp14 plays a role in sgmRNA recombination frequency and recombination patterns in virally-infected cells and virions. Using RNA-sequencing, Gribble *et al.* demonstrated that wild-type SARS-CoV-2 and MERS-CoV performed extensive recombination and generated diverse populations of both sgmRNAs and defective viral genomes (DVGs) *in vitro* [67]. Due to the lack of available proofreading-deficient nsp14-ExoN catalytic mutants in MERS-CoV and SARS-CoV-2, the authors used instead a MHV-CoV mutant with engineered ExoN catalytic inactivation for their subsequent studies. The catalytically-inactivated nsp14-ExoN in MHV-CoV generated significantly altered patterns of recombination and

decreased recombination frequency when compared to MHV-CoV wild type. Decreased sgmRNA populations and increased defective viral genomes (DVG) were also observed in MHV-ExoN(-), thus further suggesting the importance of ExoN in viral RNA synthesis and viral fitness.

These data demonstrate that nsp14-ExoN is a key determinant of both high fidelity CoV replication through its proofreading function and normal viral genome recombination. This highly-conserved multi-functional domain is therefore a vulnerable target for the development of anti-CoV therapies.

3.2. Issues with coronavirus proofreading and nucleoside analogue therapeutics

The presence of ExoN proofreading activity in nsp14 confers a >20-fold increase in replication fidelity of coronaviruses compared to other RNA viruses. This has resulted in native resistance of CoVs to many nucleoside analogue inhibitors including some that have proven antiviral properties [54,63,66]. Ribavirin (RBV; 1-β-d-ribofuranosyl-1, 2,4-triazole-3-carboxamide) is a broad-spectrum antiviral agent that is used to treat a range of viruses including Hepatitis, Human Orthopneumovirus, Lassa fever virus and Hantavirus, among others [68,69]. The monophosphate form of ribavirin inhibits inosine 5'-monophosphate dehydrogenase (IMPDH) therefore depleting the intracellular pools of GTP and attenuating viral RNA synthesis. The triphosphate form of RBV mimics adenosine and guanosine and once incorporated into the viral genome, induces viral mutagenesis due to its ability to pair equally well with uracil and cytosine [70,71]. However, in isolates of SARS-CoV *in vitro,* ribavirin was excised from the viral genome due to the proofreading activity of the nsp14 ExoN domain, suggesting a potential mechanism of resistance to some small molecule antivirals agents [66]. In another *in vitro* study while the WT SARS-CoV was resistant to both ribavirin and the mutagen 5-fluorouracil (5-FU), a pyrimidine analogue, knocking out ExoN function resulted in 300-fold greater sensitivity to the compounds, demonstrating the importance of exonuclease activity in CoV resistance to nucleoside analogue inhibitors [63].

Targeting coronaviruses with existing antiviral compounds is challenging because of the unique 3' to 5' exonuclease proofreading activity of this group of viruses. In the following sections, we review the current state of play with respect to the therapeutics that are being tested against SARS-CoV-2 in particular and that show promise in molecular studies. We also review potential viral targets based on the in-depth studies of the CoV proofreading mechanism that we have discussed, and we consider several possible methods and techniques that could be employed to deliver novel therapeutic solutions against this virus.

3.3. Proofreading as a target

The unique ability of coronaviruses to remove mismatched nucleotides during genome replication and transcription has resulted in resistance to NI drugs whose success relies on viral replication infidelity [15,54,63,66]. The proofreading mechanism that can excise non-canonical or mismatched nucleotides therefore presents an obvious target for the design of novel therapeutics against this family of viruses. Key players in coronavirus proofreading include the RdRP nsp12 protein that initially selects and incorporates nucleotides into the growing RNA chain, the 3' to 5' exonuclease function of nsp14 that excises mismatched nucleotides and the cofactor nsp10 that is proposed to stabilise the nsp14 ExoN domain [45,58].

The RNA-dependent RNA polymerases (RdRPs) of RNA viruses have thus far been one of the main targets of choice in developing treatments for a number of viral infections [72]. They play a fundamental role in RNA synthesis, and are structurally conserved, and the lack of a host homologue facilitates the design of drugs that avoid off-target effects [39]. The active metabolites of nucleoside analogue inhibitors target the viral RdRP and compete with native nucleotides for incorporation into the growing RNA strand. This phenomenon is augmented by the low fidelity of viral RdRPs; they incorporate mutations at a much higher rate than the polymerases of DNA-based organisms [73]. This has been attributed to the difference in their genome sizes; RNA virus genomes range from ~3 to ~32 kb whereas DNA genomes can be up to several hundred megabases in size and therefore require a replication machinery that is much more accurate than that of their tiny viral counterparts [74]. The comparatively large RNA genomes of coronaviruses are associated with low mutation frequencies compared with other RNA viruses [55]. However, *in vitro* studies of SARS-CoV-1 RdRP complexed with nsp7 and nsp8 (without nsp14) demonstrated that the polymerase itself is error-prone with a lower fidelity than the RdRP of dengue virus, a flavivirus with a three-fold smaller genome [66]. To rationalise the apparent contradiction between the observed low mutation frequency of coronaviruses and the low fidelity of their RdRPs, it was hypothesised that during the evolution of these viruses the acquisition of nsp14-ExoN as an active part of the replication complex to increase fidelity allowed relaxation of nucleotide selection by the viral RdRP. Indeed upon serial passaging in cultured cells, the genomes of ExoN-knockout MHV and SARS-CoV mutants accumulated up to 21-fold more mutations than their wild type controls, thus providing direct experimental evidence for a connection between ExoN activity and CoV replication fidelity [54,55]. The promiscuous substrate choice of RdRPs enables the significant incorporation of nucleotide analogues into viral RNA. Hence, a combination of virally targeted agents with a specific inhibitor of ExoN proofreading might enhance the sensitivity of SARS-CoV to antiviral treatment.

The multifunctional nsp14 protein is a key target of anti-CoV strategies not only for its role in resistance to known antivirals through its proofreading mechanism, but also for its role in several processes of

the virus life-cycle and pathogenicity [22,57,67,75]. The N-terminal ExoN domain exhibits 3' to 5' exonuclease activity that corrects nucleotide mismatches introduced by the low-fidelity RdRP [22,66]. At the C-terminus of the nsp14 protein is the N7-methyltransferase domain that functions in mRNA capping along with several other nsp proteins. In addition to facilitating recognition by the cellular translation machinery, capping viral mRNAs protects them from host antiviral responses that recognise uncapped RNAs as "non-self" [45,46]. Mutations specifically abolishing the N7-MTase activity in West Nile Virus were shown to have a clear detrimental effect on replication, demonstrating the importance of this enzymatic function of nsp14 [76]. A study that developed a high throughput fluorescence assay to identify inhibitors of the nsp14-N7-MTase detected 20 compounds with a range of specificities against both viral and cellular MTases [77]. These compounds provide a first step towards the development of more specific inhibitors of viral methyltransferases and demonstrate an alternative strategy for targeting nsp14. Another protein involved in viral cap formation is the nsp16 2'O-MTase that mediates the final step in cap synthesis [48]. This protein requires direct interaction with the nsp10 cofactor for its activity. Intriguingly, nsp10 is also a cofactor for the nsp14 ExoN activity although it is not required for the nsp14 role in mRNA capping [19]. The nsp10 cofactor could stimulate nsp14 exoribonuclease activity >35-fold compared with nsp14 alone highlighting the importance of this protein interaction [65]. A reverse genetics approach identified key residues on an nsp10 surface involved in the interaction with SARS-CoV-1 nsp14 ExoN and important for the latter's activity and for viral replication. These residues also appeared to be part of the nsp10 domain that interacts with nsp16. Hence, nsp10 possesses overlapping interaction surfaces for the activation of two important viral enzymes [19]. Targeting nsp10 could simultaneously disable several functions that are crucial for viral success. In addition to proofreading, genome recombination and mRNA capping, the multifunctional nsp14 protein has been implicated in the modulation of the host innate immune responses. The introduction of a specific mutation in the zinc finger 1 of an *alphacoronavirus* nsp14-ExoN domain produced a viable virus that triggered a decrease in accumulation of dsRNA intermediates and a reduced antiviral response and apoptosis in comparison to the wild type virus, indicating that CoV nsp14 plays a role in modulation of the innate immune response [75].

Taken together, these data demonstrate the vital functions that the nsp14 protein and its cofactor nsp10 play in viral replication and pathology and in combination with strategies that target the replication-transcription mechanism, notably RdRP, these nsps present vulnerable targets for anti-SARS-CoV-2 therapies.

Genomics-based systems present a highly efficient method to specifically knock out a target protein such as nsp14, and these techniques will be discussed later.

4. Potential anti-SARS-CoV-2 strategies targeting replication fidelity

4.1. Repurposing existing antiviral drugs

To date, there are no approved vaccines or antiviral drugs against human or zoonotic CoVs although the SARS-CoV-2 pandemic is fueling drug discovery and trialling on an unprecedented scale. Repurposing existing antiviral drugs, both approved and experimental, to fight SARS-CoV-2 has been widely recognized as the quickest strategy to obtain effective therapies to control the COVID-19 outbreak [78]. Here we review some key antiviral NI drugs that are being examined for anti-SARS-CoV-2 activity and have shown promising results.

4.1.1. Remdesivir (GS- 5734^{TM})

Remdesivir (RDV) an experimental drug, also known as GS-5734 was initially developed by Gilead Sciences to treat Ebola and related viruses. GS-5734 is a prodrug that is metabolised into its active form, GS-441524, an adenosine nucleotide analogue that is effective in blocking *in vitro* and *in vivo* replication of Ebola virus. The active compound targets the viral RdRP enzyme and interrupts RNA genome synthesis [79]. It has shown broad-spectrum *in vitro* and *in vivo* activity against a range of RNA viruses including members of the *Filoviridae*, *Paramyxoviridae* and *Pneumoviridae* families [80]. Subsequent *in vitro* and animal studies demonstrated antiviral activity of RDV against CoVs including SARS-CoV and MERS-CoV [81–84] and SARS-CoV-2 [85]. Lo *et al.* also demonstrated that the combination of remdesivir and the HCV protease inhibitor simeprevir synergistically suppresses SARS-CoV-2 replication *in vitro* [86].

RDV exerts its effect firstly by competing with ATP for the binding to RdRP and then attenuating replication after incorporation into the growing RNA molecule by delayed chain-termination. Using *in vitro*-expressed active complexes of the SARS-CoV-2 RdRP (nsp12) with cofactor nsp8, Gordon *et al.* demonstrated that the active form of RDV displays high selectivity over its natural counterpart, ATP [38]. In addition, the chain termination mechanism of RDV is not ‘classical’ but results in termination after a further incorporation of up to three nucleotides at position i+3 [87]. RDV has a normal ribose 3’-OH that presumably allows incoming nucleotides to effectively attach until a steric clash of the ribose 1’-CN moiety of RDV with residues in the RdRP protein as the RNA chain moves through the polymerase and prevents any further nucleotide incorporation. This aspect of the inhibitory mechanism of RDV has important implications for the proofreading activity of coronaviruses; the presence of up to three correctly paired canonical nucleotides at the end of the RDV-terminated transcript may protect the incorporated RDV from ExoN-mediated excision. However, a mutant of the murine hepatitis virus (MHV) lacking nsp14 ExoN proofreading was significantly more sensitive *in vitro* to RDV than the WT virus, and RDV resistant mutants recovered through virus passaging were also

more sensitive to the drug, albeit with reduced pathogenicity, demonstrating that ExoN in addition to RdRP, plays a role in virus resistance to this drug [54,88].

To identify the crucial amino acid residues important for nucleotide recognition, discrimination and excision of RDV, Shannon *et al.* derived models of catalytically-competent SARS-CoV-2 RdRP and ExoN enzymes using available structures of related viral proteins [39]. They then mapped the active metabolite of RDV (GS-441524) to the active sites of the enzymes and demonstrated that the predicted mechanism for the drug's incorporation, chain termination and altered excision due to the ribose 1'-CN group were supported by the models. The study suggested that once incorporated, the active form of RDV would distort and force its ribose moiety to occupy the space needed for an efficient two metal ion catalysis to happen at the active site of ExoN.

In another recent study, polymerase assays using SARS-CoV-2 RdRP complexed with nsp7 and nsp8, and bound to a template-primer RNA molecule demonstrated inhibition upon the addition of RDV-TP [37]. Thus far, RDV, an antiviral originally designed against Ebola, looks to fit the criteria for an anti-CoV therapeutic; selective incorporation into RNA strands by RdRP and some resistance to excision by ExoN.

4.1.2. β-D-N4-hydroxycytidine (NHC)

β-D-N4-hydroxycytidine (NHC; EIDD-1931), a cytidine analogue with a modified nucleobase, has antiviral activity against a broad spectrum of viruses including coronaviruses such as HCoV-NL63, SARS-CoV and other viruses including HCV, influenza A and B viruses, alphaviruses and Ebola virus [89–95]. A recent *in vitro* study demonstrated that NHC effectively inhibits two different β-CoVs; murine hepatitis virus (MHV) and MERS-CoV with only slight toxicity and low frequency of resistance [96]. NHC antiviral activity is likely in part due to incorporation of the molecule in the growing RNA strand and mismatches during subsequent replication and transcription resulting in lethal mutagenesis of the viral genome [52] although the detailed mechanisms of NHC antiviral activities are yet to be discovered. NHC is the first mutagenic NI demonstrating the ability to evade active ExoN proofreading. WT MHV and ExoN(-) MHV were inhibited with similar sensitivity unlike other antivirals tested, for example RDV, that displayed increased potency in the absence of an intact ExoN [88]. One possible explanation for this observation is that NHC is not recognised, targeted and excised by ExoN. However, it has also been proposed that NHC may inhibit viral replication by additional mechanisms beyond inducing lethal mutagenesis. Previous reports already suggested that NHC might alter RNA secondary structure or block the virion release process [89,94]. Of note, it has recently been reported that NHC effectively targeted different zoonotic coronaviruses including SARS-CoV-2 in both *in vitro* and animal models, with an enhanced efficiency against coronavirus containing

resistance mutations to RDV [97]. These results imply that NHC shows promise as an antiviral treatment for multiple viruses including SARS-CoV-2.

4.1.3. Galidesivir (BCX4430)

Galidesvir is an adenosine analogue that has been developed for treatment of multiple virus infections including the Filovirus and Ebola [52]. In the infected cells, this prodrug is rapidly converted into its nucleotide triphosphate derivative that functions as an RNA chain terminator, once the active monophosphate form is incorporated into the nascent RNA by RdRP [98]. BCX4430 has two structural modifications compared with its native nucleoside ATP; 1) a C-glycosidic bond replaces the usual N-glycosidic bond, and; 2) there is an imino group in place of the furanose oxygen [99]. In template-directed primer extension assays, Hepatitis C virus RdRP-driven RNA synthesis was prematurely terminated by BCX4430 in a delayed chain termination manner, two bases after incorporation of the nucleoside analogue at position i+2 [100]. This is consistent with other observations that nucleoside analogues with an intact 3'-OH may still incorporate subsequent bases until steric clashes with incoming bases or residues of the polymerase complex terminate chain elongation [38]. Beside Filoviruses, Galidesvir demonstrated activity against CoVs such as MERS-CoV and SARS-CoV *in vitro* [98,100].

4.1.4. Favipiravir (T-705)

Favipiravir (T-705; 6-fluoro-3-hydroxy-2-pyrazinecarboxamide) was first developed as an anti-influenza virus therapy targeting the viral RdRP and subsequently has demonstrated broad spectrum antiviral activity [101–103]. Favipiravir is a purine nucleoside analogue precursor with a modified nucleobase that is converted into the active form, T-705-RTP (T-705-4-ribofuranosyl-5-triphosphate), by a cellular enzyme, and then disrupts viral RNA replication as the monophosphate form is incorporated into the growing chain instead of guanosine- and adenosine-monophosphates. There are two proposed mechanisms of antiviral action of T-705; the first is non-obligate chain termination where RNA synthesis terminates despite the compound containing an intact 3'-OH [104,105]. The second proposed mechanism is the incorporation of multiple molecules of T-705 into the growing RNA chain and subsequent mismatch pairing leading to lethal mutagenesis of the virus [106]. However, it has been hypothesised that the increased mutation rates observed in response to T-705 treatment could also be due to a biased cellular nucleotide pool in the presence of excess T-705-RTP and properties of the viral RdRP. This latter theory could explain the apparent contradiction between the chain termination and lethal mutagenesis theories [107].

4.1.5. Sofosbuvir

Sofosbuvir is a licensed nucleotide analogue for the treatment of hepatitis C virus (HCV) infection. Sofosbuvir, a prodrug, is metabolised in the liver to produce the active triphosphate form, β-d-2′-deoxy-2′-α-fluoro-2′-β-C-methyluridine triphosphate (2′F-2′C-Me-UTP). The compound acts as an HCV RdRP (NS5B) inhibitor which can insert to the RdRP active domain, incorporate into the nascent RNA strands, thereby blocking chain extension [108,109]. Despite the presence of an intact 3'-hydroxyl group predicted to incorporate an incoming NTP and thereby continue RNA elongation, sofosbuvir acts as a chain terminator and attenuates viral RNA synthesis. It is thought that this is due to the presence of the 2′F-2′C-Me group in the potent triphosphate form of sofosbuvir that leads to steric clashes with the incoming NTP and termination of HCV RNA synthesis [110]. Apart from HCV, sofosbuvir has been shown to significantly inhibit other Flaviviruses such as Zika virus, Dengue, and West Nile virus [111–113]. Sofosbuvir is one of several antiviral compounds that is predicted to bind tightly to SARS-CoV-2 RdRP in molecular modelling experiments, suggesting potential inhibition strategies against the newly emerged coronavirus [40]. A study using polymerase extension assays with a pre-annealed RNA template and primer and a pre-assembled nsp12/nsp8/nsp7 complex demonstrated that sofosbuvir in its active form could block chain extension [114]. Whether ExoN can excise this, and other, experimental drugs *in vitro* or *in vivo* remains to be tested.

4.2. Designing novel nucleoside analogue inhibitors

Due to the obligate intracellular nature of viruses, finding drugs that target the virus but do not harm the host cell is challenging. Unlike other antimicrobial drugs (for example, antibiotics), antivirals do not directly destroy viruses; they function by inhibiting replication at various stages of the virus life-cycle. Antiviral nucleoside analogues specifically target the viral DNA and RNA replication machinery, and this mechanism makes them potential inhibitors of host nucleic acid synthesis as well, especially mitochondrial DNA and RNA synthesis [115]. Ideally, a successful NI-based antiviral therapy should selectively target the viral genome, be effective at low micro molar concentrations to minimise cellular toxicity and demonstrate a high barrier to resistance. The structural conservation of the polymerase binding sites targeted by NIs is high among virus families and therefore mutations that confer NI resistance often result in diminished virus fitness. In this respect NIs are good antiviral candidates as they demonstrate a relatively high barrier to resistance emergence [116]. The capacity of the nsp14 ExoN proofreading machinery to recognise and excise non-canonical nucleotides during replication and transcription presents a major obstacle to the success of NI activity against coronaviruses. The broad-spectrum NI ribavirin that has been used successfully against a range of viruses is readily incorporated by coronavirus RdRP into the growing chain; however, it is just as readily excised out again by the proofreading activity of nsp14-ExoN [66]. Structural and mechanistic studies are uncovering the nucleotide substrate requirements for the ExoN domain to effectively carry out its

proofreading function and these findings are revealing the mechanism behind the success, or indeed the failure, of some NIs to inhibit coronavirus replication. For the coronavirus ExoN to effectively excise a nucleotide from the 3' end of the RNA chain, the relevant nucleotide should display a mismatch with the complementary template nucleotide, presumably mimicking an erroneous replication event. In addition, the nucleotide needs an intact ribose-3'-OH moiety for excision to occur; *in vitro* studies demonstrated that ExoN cannot excise 3'-modified nucleotides as nucleotides carrying 3'-puromycin or 3'-phosphate resisted excision [65]. The NI ribavirin has both an intact 3'-OH and a modified nucleobase that mimics both adenosine and guanosine [70,71]. The base pairing of this NI with either uracil or cytosine may resemble a mismatch to the ExoN machinery which then efficiently removes it from the growing RNA chain.

CoV RdRps are able to tolerate a wide variety of different chemical modifications of NIs and incorporate them into the RNA strand [52]. The ability of the ExoN machinery to recognise these varied modifications is, therefore, a key area of investigation towards the design of new NI analogues.

The requirements of an effective NI antiviral compound that can evade the ExoN proofreading excision mechanism can be summarised into two main strategies;

1) The incorporated NI should escape detection by the ExoN excision mechanism altogether leading to the assimilation of multiple NIs in the RNA strand that then introduce mutations to the viral genome through mismatching during replication resulting in lethal mutagenesis. β-D-N4-hydroxycytidine (NHC) and Favipiravir, which both carry modified nucleobases, are examples of NIs that may act through this mechanism [94,106].

2) The NI bears a modification that leads to chain termination**.** There are several mechanisms by which NIs can evade proofreading by the second strategy. Chain termination may be immediate or may occur following a limited extent of continued RNA or DNA synthesis (delayed). It may be obligate or non-obligate. Obligate chain terminators lack the ribose-3'-OH moiety required for polymerisation of the next NTP and once incorporated, block further synthesis [117]. The 3'-OH is also required for excision by ExoN so obligate chain terminators cannot be removed by the proofreading mechanism [65]. Several promising NIs against coronaviruses (RDV, BCX4430, T-705, Sofosbuvir) lead to non-obligate chain termination; they have a 3'-OH but the various modifications they carry are presumed to result in steric clashes either with the next incoming NTP or with amino acid residues in the active site of RdRP as the incorporated NI moves through the protein complex [38,98,106,108,109]. Knowledge of the structural modifications to NIs that are effective in evading ExoN proofreading will point the way to the design of novel molecules.

Molecular modelling; theoretical and computational methods to model or mimic the behaviour of molecules; continues to be an important strategy for developing novel NIs. Understanding the virus target-ligand interactions is a key challenge. The COVID-19 Docking Server (https://ncov.schanglab.org.cn/), a free and interactive tool, has recently been introduced; this is a web server that predicts the binding modes between COVID-19 targets and potential ligands including small molecules, peptides and antibodies. Structures of proteins involved in the virus life cycle have been gathered where available or constructed based on the homologs of coronavirus, and uploaded ready for online docking experiments. This, and other emerging online tools, will greatly facilitate the discovery of novel small molecule anti-SARS-CoV-2 therapeutics.

4.3. Novel gene therapies for SARS-CoV-2 treatment

Gene- or nucleic acid (NA)- based therapies involve the use of synthetic NA molecules as tools or therapeutic agents for disease intervention by modulating the targeted gene expression. NA therapeutics is considered to be one of the most important drug discovery platforms in addition to small molecules and antibodies therapeutics. Current therapeutic nucleic acids (TNA) include antisense oligonucleotides (ASO), CRISPR-Cas and other gene-editing tools, small interfering RNA (siRNAs), ribozymes, aptamers and more [118]. These molecules modulate gene expression either by RNA interference, catalytic cleavage of the transcripts or by binding to specific peptide domains [119]. The use of synthetic NA molecules was described for the first time forty years ago in a study that demonstrated oligonucleotide-mediated inhibition of Rous sarcoma viral RNA translation [120,121]. Since then, NA therapies have been developed for treatments such as cancer, central nervous system disorders and virus infection. Despite their early discovery, few TNA-based drugs were approved by the FDA until recent years when improved target specificity, efficacy and NA delivery was demonstrated [122].

The high mutation rate of SARS-CoV-2 could potentially complicate the discovery of conventional antiviral drugs and preventative vaccines that are usually time-consuming to develop and clinically evaluate. Nguyen et al. recently analysed the SARS-CoV-2 genetic sequences from patients in three different countries (USA, China, Australia), and found several single-nucleotide polymorphism (SNP) that were proposed to enable cross-species transmission from bats to humans. This suggested that SARS-CoV-2 actively acquires novel genetic changes and this could potentially impact the current treatment strategies and allow the virus to develop antiviral resistance [123].

An antiviral oligonucleotide approach might be worth exploring as a therapeutic agent due to its high specificity, affinity and relatively simple rational design of oligonucleotide against SARS CoV-2. TNAs could be potentially used for modulating the expression of genes encoding proteins that contribute to

the proofreading mechanism in SARS-CoV-2, thereby attenuating the viral replication more effectively unlike the conventional small molecule approaches.

In this section, the uses and the mechanism of actions of several promising NA therapeutics to treat coronaviruses, such as Antisense Oligonucleotides (ASO), small interfering RNAs (siRNA) and RNA targeting-CRISPR systems will be reviewed.

4.3.1. Antisense oligonucleotide technology

Antisense oligonucleotides (ASOs) represent a promising class of compounds that can be developed quickly and specifically against SARS-CoV-2. ASOs are single DNA strands containing around 20 nucleotides which specifically hybridize to the complementary sequence of the target RNA. Depending on the type of ASO, the resulting DNA:RNA hybrid can either induce mRNA cleavage catalyzed by the host RNase H enzyme, alter splicing or form a steric blockade to disrupt translation, thereby knocking down the expression of target proteins [124]. Notably, due to their resistance against various enzymes including nucleases, ASOs can be stable for hours in both cellular and extracellular environments which makes ASO highly applicable to *in vivo* models and clinical applications [125,126]. There have been seven FDA-approved ASO disease intervention therapeutics, including Vitraven (cytomegalovirus retinitis, 1998) and Exondys 51 (Duchenne muscular dystrophy, 2016) [127].

A number of antiviral ASOs have been designed to inhibit respiratory viruses such as Influenza A, H7N7, H5N1, H3N8 and RSV [128–135]. Specifically, phosphorodiamidate morpholino oligomers (PMO), a morpholino-type ASO, have also been explored in the treatment of various RNA viruses, including Ebola virus, flaviviruses, coronaviruses and picornaviruses [126]. PMO primarily exerts antiviral activity in RNaseH-independent manner by base pairing to the target RNA and sterically inhibiting the RNA processing, subsequently shutting down the RNA translation or potentially viral RNA replication [126]. PMO- antisense oligos are frequently designed to bind to either the translation start site (AUG) region, the 3'-terminal region or transcription regulatory sequences (TRS) of targeting mRNA and RNA genome segments. The potency of PMO antisense targeting the 5 'end of MHV genome in viral inhibition has been demonstrated by interrupting the translation process of the viral replicase polyprotein [136]. These ASOs were shown to effectively enter host cells, and decrease coronavirus growth and proliferation [136]. Further study also demonstrated improved antiviral efficacy of morpholino-type ASOs which specifically target the transcription regulatory sequences (TRS) located in the 5’ untranslated region (UTR) of MHV genome, leading to blockage of SARS-CoV replication [137]. Additionally, phosphorothioate ASOs were demonstrated to be effective in downregulation of SARS-CoV with up to 90% inhibition of the target structural proteins (envelope, membrane, nucleocapsid) in cell lines infected with coronavirus [138]. Another study showed that

phosphorothioate ASOs targeting the coronavirus spike protein complexed with beta-cyclodextrin exhibited greater antiviral activity than unbound phosphodiester ASOs at 25 μM *in vitro* [139]. The effectiveness of the ASO strategy in prophylactic treatment for MHV was also demonstrated by a reduction in the effects of the virus [140].

Beside targeting directly the virus, ASOs have also been designed to suppress host cellular enzymes which play vital roles in virus infection [126]. It was shown that transmembrane protease serine 2 (TMPRSS2) was crucial for stimulation of viral proteins related to membrane fusion during viral entry into host cells in influenza, para-influenza and coronavirus infections including SARS-CoV-2 [141–143]. Altered splicing of TMPRSS2 pre-mRNA induced by ASOs caused production of non-functional TMPRSS2 enzyme, leading to decrease of viral titers by 2 to 3 log(10) units [144]. Therefore, developing an ASO that “mis-regulates” the splicing of TMPRSS2 or other host proteins involved in essential viral activities could present a highly effective antiviral treatment.

Along with proven effectiveness and specificity, ASO inhibition is not predicted to be targeted by the CoV proofreading machinery allowing ASO to become a promising therapeutic strategy to combat SARS-CoV-2. The 5’UTR regions or transcription modulatory sequences of the SARS-CoV-2 RNA genome or RNA replication could be used as targets for ASO development. In addition, ASOs could also be designed to target different mRNA and RNA genome segments encoding for key proteins in replication machinery such as nsp8, nsp12, nsp10, nsp14 to shut down their expression and thereby inhibit viral replication. By combining several ASOs against multiple targets, crucial functions such as infectivity, RNA replication, virion release could be disrupted and subsequently attenuate the viral life cycle more effectively.

Crucially, ExoN in nps14 could be a promising therapeutic target for ASO therapy, by specifically knocking down the nsp14 gene expression and inhibiting the proofreading machinery in coronavirus. A combination of ASOs targeting nsp14 gene expression and antiviral drugs such as ribavirin and remdesivir could be explored and the antiviral activity of these drugs could potentially be restored; previously they were shown to be ineffective due the ExoN activity in nsp14. Recently, Rocchi et al. have demonstrated that lipid-modified ASOs (LASOs) have the ability to self-assemble into 11nM “nanomicelles”, allowing ASO to enter into the cells without the need for a transfecting agent [145–147]. These physicochemical properties could allow hybrid nanomicelle formation carrying different active sequences against essential proteins that could be administered by aerosol. Furthermore, Rocchi et al recently found that these nano-structures can encapsulate hydrophobic chemotherapeutics and promote their delivery [148]. They showed that chemotherapy encapsulation in LASO nanocomposites, allows a greater solubility of the compound in water and greatly increases the efficacy of the compounds. According to the European Medical Agency, Gilead’s remdesivir is

poorly soluble in water, and requires betadex sulfobutyl as a solubilizing agent [149]. Thus, encapsulation of nucleoside compounds such as RDV and RBV in antiviral LASO nanocomposites may serve as combinatorial therapy to restore sensitivity to nucleosides analogues or other antiviral treatments (**Figure 4**).

4.3.2. Small interfering RNA (siRNA)

siRNAs have been widely used for silencing gene expression in biological research and antiviral pathogenesis. siRNA technology takes advantage of the cellular RNA interference (RNAi) pathway that uses small molecules of RNA to interact with complementary mRNAs and subsequently inhibit the expression of a particular gene [150]. Synthetic 21-23 nucleotide (nt) double stranded RNA molecules designed to target the gene of interest are recognized by the host RNA-induced silencing complexes (RISC) which then separate the two strands of RNA. The RISC-associated antisense strand guides the complex to the specific target sequences, leading to their cleavage and degradation (**Figure 5**) [151,152]. siRNAs have been targeted to various viruses including HIV, HBV and SARS-CoV and shown to inhibit virus replication in the infected host cells [153]. siRNA designed to specifically target the spike protein S gene demonstrated effective and specific inhibition in SARS-CoV-infected cells [154]. Another study showed that siRNA against either the 3'-UTR or sequences encoding for structural proteins can block SARS-CoV replication in cell culture [155].

The RNA-dependent RNA polymerase (RdRP) plays a key role in the replication of the virus and represents an interesting target to inhibit virus proliferation. One approach based on plasmid-mediated siRNA was used to target viral RNA polymerase. The titer assays and protein level examination of SARS-CoV infected cells demonstrated the inhibition of viral replication leading to an effective blocking of virus expansion [156]. Another study targeted the RdRP protein of SARS-CoV using short hairpin RNA (shRNA), a siRNA precursor, via plasmid expression in SARS-CoV Vero E6 infected cells [157].

The structural proteins of coronaviruses have been targeted by siRNA methods in a number of studies. He *et al.* reported that a combination of siRNAs inhibiting two structural proteins simultaneously in SARS-CoV infected cells displayed a synergistic effect on reducing viral accumulation even at very low concentrations [158]. Ankerström *et al.* targeted accessory proteins 7a/7b, 3a/3b and the structural protein S gene by siRNA [159]. In SARS-CoV infected Vero E6 cells, a significant decrease of virus yield was observed when expressing each siRNA separately. Additionally, the siRNAs specifically inhibited the gene they were targeted to; related sequences with only two or more mismatches (17/19bp) were not inhibited. This demonstrates the high level of specificity of the technique [159]. The use of siRNAs has also been reported for the inhibition of

Human coronavirus NL63 (HCoV-NL63). Pyrc *et al* demonstrated that siRNAs targeting the S gene inhibited early stage viral infection with high potency and low cytotoxicity [91].

Another approach for preventing viral entry would involve targeting the cellular receptors required for the virus-cell recognition. Lu *et al.* developed an RNAi construct for silencing the angiotensin-converting enzyme 2 (ACE2) that is involved in the SARS-CoV penetration into host cells [160]. Despite the study showing reduced SARS-CoV replication in ACE2-silenced Vero E6 cells, the mechanism of SARS-CoV viral entry will need further investigation due to adverse effects and only modest viral inhibition via ACE2 silencing [161].

Several antiviral siRNAs have undergone clinical trials, including TKM-Ebola targeting multiple transcripts (L, VP24, and VP35) of EBOV (phase I) [162], ALN-RSV01 for RSV targeting its structural S protein (phase II) [163]. Preclinical tests on rhesus macaques infected with SARS-CoV were carried out with two siRNA targeting respectively the Spike protein gene and nsp12 RdRP. The results demonstrated an effective activity either by prophylactic or therapeutics treatment. A reduction of damage in alveoli was observed and the accumulated dosage (10 to 40 mg/kg) of siRNA did not induce any toxicity [164,165].

4.3.3. RNA-targeting CRISPR systems

Genome editing mediated by the Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-Associated protein 9 (CRISPR/Cas9) technology has been gathering pace as an innovative strategy to combat virus infection in humans. CRISPR/Cas9 is one of the most frequently used RNA-guided DNA endonuclease systems that determines target sites for dsDNA cleavage via RNA-DNA hybridization between guide RNA (gRNA) and specific DNA target sequences [166–168]. CRISPR/Cas9 as an antiviral agent can exert its effects through either targeting directly the viral genome or disrupting the expression of host proteins involved in viral crucial activities [169]. The canonical CRISPR/Cas9 system can target dsDNA viruses such as herpesviruses, human papillomavirus (HPV), Hepatitis B virus (HBV) or RNA viruses which have dsDNA intermediate during life cycle such as HIV [170]. Numerous studies demonstrated that the use of CRISPR/Cas9 targeting long terminal repeat (LTR) promoter regions or key viral genes of HIV showed potential antiviral effects such as inhibition of HIV-1 production and infectivity in $CD4^+$ T cell-derived cell lines [169].

Although the CRISPR-Cas9 system was thought to recognise DNA exclusively, studies have demonstrated a strategy to engineer CRISPR/Cas9 systems to cleave RNA directed by RNA-targeting guide RNA (rgRNA), for more programmable and site-specific cleavage of single stranded RNA (ssRNA) targets [168,169]. A natural Cas9 endonuclease from *Francisella novicida* (FnCas9), for instance, was shown to inhibit the recombinant Hepatitis C virus (HCV) in hepatocellular carcinoma

cells (Huh-7.5) via reduction of the viral protein expression [171,172]]. A recent discovery of the RNA-targeting CRISPR-Cas13d system based on the RNA-guided RNA endonuclease derived from *Ruminococcus flavefaciens* XPD3002 has opened up tremendous potential therapeutic opportunities to combat RNA viruses [173,174]. The guide RNA (gRNA) or CRISPR-associated RNAs (crRNAs) consisting of a customizable 22-nt spacer sequence leads the associated Cas13d to the RNA target for RNA cleavage and gene silencing [175]. Nguyen *et.al* have recently presented a strategy to combat SARS-CoV-2 based on the CRISPR/Cas13d system [123,176]. Accordingly, the cleavage of SARS-CoV-2 genome can be achieved by using a Cas13d protein and guide RNAs (gRNAs) consisting of spacer sequences complementary to the target sequences. Crucially, the gRNAs were designed to specifically recognize the viral RNA genome, thus avoiding disruption to the human transcriptome. The authors designed 10,333 potential gRNA targeting 10 coding regions of the SARS-CoV-2. As a safe and effective viral vector for gene therapy in clinical experiments, adeno-associated virus (AVV) was considered as a vehicle to administer the Cas13d effector to the patients [123,176]. Due to the small size, the Cas13d effector can be packed in the same AVV particle along with up to three different gRNAs, resulting in high efficacy, specificity, and presenting a high barrier to resistance (**Figure 6**).

Recently, a prophylactic antiviral CRISPR-based system has been explored as a genetic tool to inhibit SARS-CoV-2 and influenza A virus (IAV) [175]. Using a bioinformatics pipeline, the authors identified highly conserved viral sequences across multiple SARS-CoV-2 genomes and designed potential CRISPR RNAs (crRNAs) for targeting these regions by CRISPR-Cas13d. The conserved regions encoded SARS-CoV-2 RdRP and nucleocapsid proteins. The Cas13d system was demonstrated to effectively recognize and degrade synthetic RNA fragments from the genome of SARS-CoV-2 and to inhibit H1N1 IAV infection in human lung epithelial cells [175]. Further studies are required to identify safe and potent CRISPR-based antiviral strategies for treating SARS-CoV-2 infections.

5. Summary

The 21st Century has seen several outbreaks of serious zoonotic coronavirus diseases; SARS-CoV-1 in 2002/3, the ongoing MERS-CoV that started in 2012 and the current SARS-CoV-2 global pandemic. These diseases have challenged scientists to understand the mechanisms by which the causal agents emerge, adapt and replicate, and have expedited drug discovery on an epic scale.

There have been significant achievements in the field of coronavirus RNA biology and these are revealing the sophistication of the genome replication and transcription processes and the highly regulated mechanisms that coordinate synthesis, fidelity, processing and repair. The ExoN 3’ to 5’ exonuclease activity is a key player in a number of crucial processes in the life-cycle of these viruses,

and it has rendered several previously successful antiviral compounds ineffective against coronaviruses. ExoN is both structurally and functionally conserved across CoVs and this factor, coupled with the absence of redundancy of function in the viral genome, makes ExoN a vulnerable target for anti-CoV strategies.

The most recent SARSr-CoV disease, COVID-19, has emerged at a time in molecular biology when post-genomics technologies are rapidly developing in terms of sophistication and target specificity. ExoN represents a logical therapeutic target of novel genomics technologies. Inhibiting ExoN activity by these nucleic acid-based approaches while simultaneously treating with conventional NIs could enhance the effectiveness of the NIs and accordingly reduce viral replication fidelity to attenuate disease. Using a therapeutic strategy like this, which combines drugs that have distinct modes of action or that interfere with different processes during viral replication may also increase the barrier to drug resistance.

It is highly likely that, in the future, novel zoonotic CoVs will emerge from virus pools in reservoir animals. Consequently it is vital to understand the molecular mechanisms by which these complex viruses replicate and maintain genome integrity while sustaining the population diversity needed for evolution and adaptation to human hosts. It is critical that this knowledge is then applied to the development of novel broad-spectrum antiviral strategies directed against conserved molecular targets and functions.

6. Conflict of interest

No potential conflict of interest was reported by the authors.

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Figure legends

Figure 1. Single-stranded RNA (ssRNA) genome of SARS CoV-2. Two-thirds of the genome encodes two large polyproteins pp1a and pp1ab that are converted into non-structural proteins (Nsp1 to Nsp16) after cleavage. The last one third of the genome encodes structural and accessory proteins. Created with BioRender.

Figure 2. Virus Replication Mechanism. To enter in the host cell, firstly the SARS-CoV-2 virus binds to the ACE2 receptor (1) to initiate the internalization (2), the vacuole containing the virus is then internalized (3) and the membrane fuses with the virus (4) in order to release it (5) into the cytoplasm of host cell. The genome is then translated to produce the polyproteins pp1a and pp1ab (6), which are cleaved by proteases (7) to yield the 16 nsps that form the RNA replicase–transcriptase complex (8). Viral genome is duplicated and mRNA encoding for structural proteins are transcribed (9). Then the subgenomic mRNA are translated into structural proteins (10).The formation of the new virion takes place on modified intracellular membranes that are derived from the rough endoplasmic reticulum (ER) in the perinuclear region (11). The new virion is then released (12). In red are localized the sites of action of a number of small molecule antivirals. Created with BioRender.

Figure 3. Model of the core replication and proofreading complex of SARS-CoV-2. Nsp12-RdRp, Nsp14-Exon, and Nsp10 are involved in the proofreading mechanism. Nsp13-helicase, an unknown GTPase, Nsp14-N7-methyltransferase and the Nsp16-2'-O-methyltransferase/Nsp10 complex are involved in the capping mechanism**.** Created with BioRender.

Figure 4. ASO technology in therapy against SARS-CoV-2. Antisense oligonucleotides (ASOs) can be conjugated with a carrier that allows delivery into the cells (1). Lipid-modified ASOs (LASO) display the ability to self-assemble into nanomicelle (2) which subsequently are encapsulated with an antiviral chemo such as RBV, RDV (3). These nanomicelles are able to enter the cell without any transfection agents (4). Once reaching inside the cell, ASO, LASO and the encapsulated drugs can be released (5). The intracellular ASOs/LASOs match to their complementary sequences (6), leading to either genome degradation (through RNaseH activity) or replication/transcription/translation blocks due to steric block forming. The antiviral compounds such as ribavirin or remdesivir can interfere with the RNA replication and transcription by targeting RdRp as described above (7). Created with BioRender.

Figure 5. siRNA technology in therapy against SARS-CoV-2. Small interfering RNA (siRNA) molecules are associated with carriers which allows their delivery into the cells (1). The siRNAs are recognized and loaded into the RNA induced silencing complexes (RISC) which separates two

strands of associated siRNA, and releases the sense strand (3,4). The RISC-associated antisense directs the complex to the target matching RNA sequence (5), which facilitates viral genome cleavage or suppression of viral replication/transcription/translation (6). Created with BioRender.

Figure 6. RNA-targeting CRISPR systems for SARS-CoV-2 inhibition. The Adeno-associated virus (AVV) serves as a carrier to deliver vector construction consisting of both Cas13d effector and gRNAs (1). In the cell, Cas13d protein and gRNA are expressed and Cas13d protein forms a complex with gRNA (2).The spacer sequence acts as a guide to the Cas13d effector by matching to their complementary viral RNA genome (3), thus allowing the associated Cas13d effector to cleave the viral RNA (4). Created with BioRender.

Figures

Figure 1. Single-stranded RNA (ssRNA) genome of SARS-CoV-2

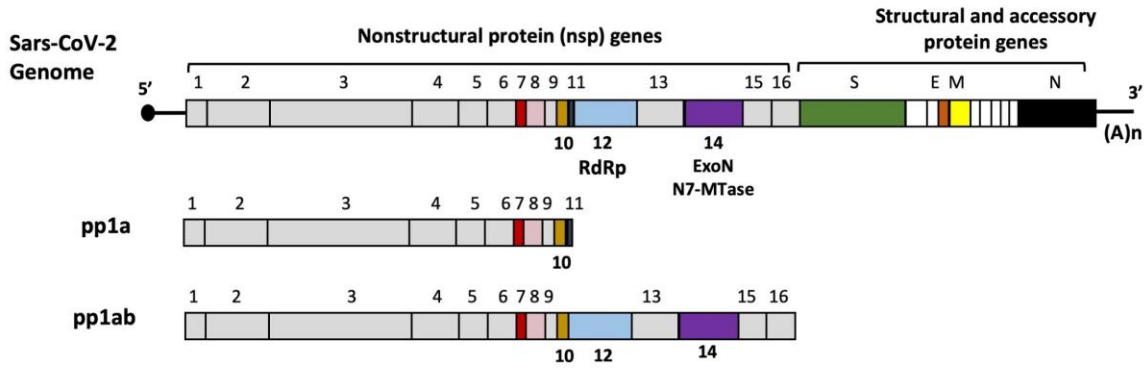


Figure 2. Virus Replication Mechanism

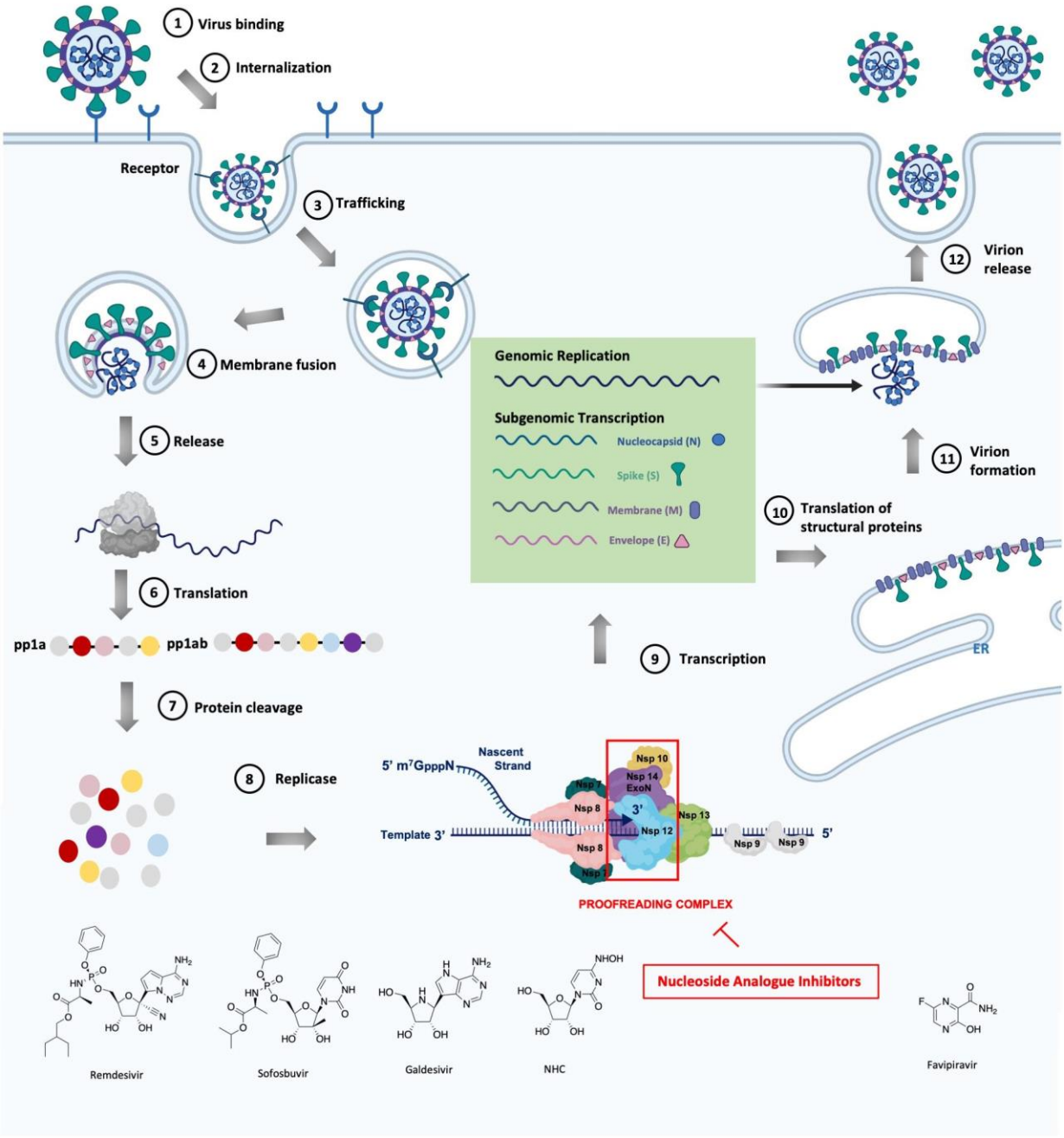


Figure 3. Model of the core replication and proofreading complex of SARS-CoV-2

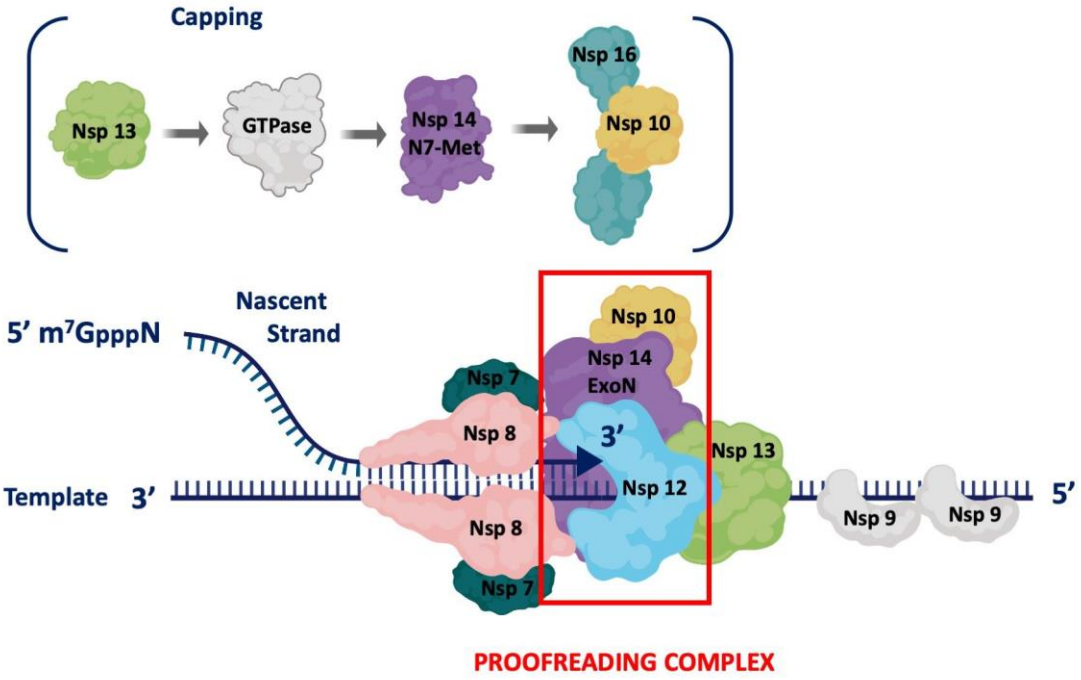


Figure 4. ASO technology in therapy against SARS-CoV-2

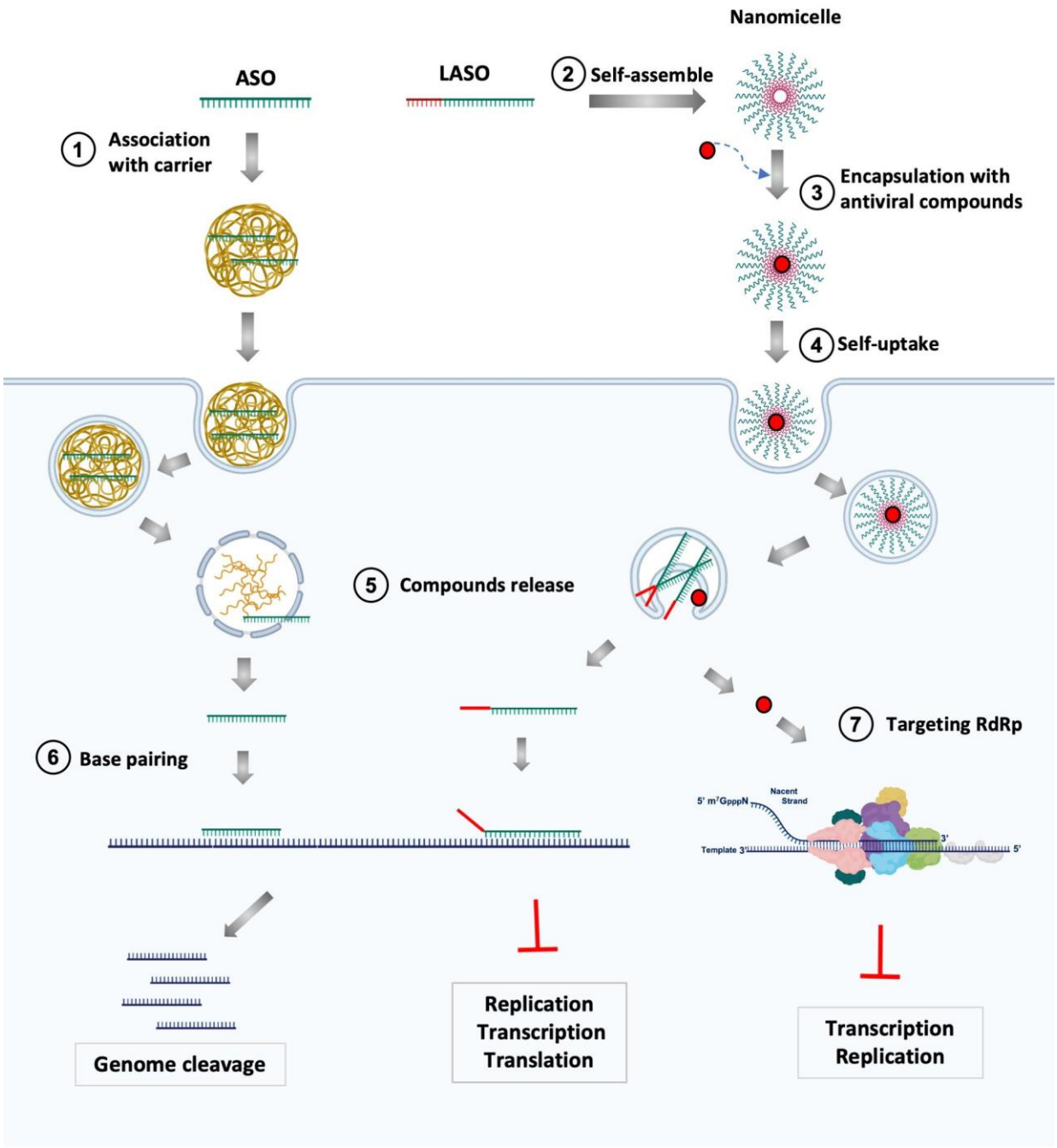


Figure 5. siRNA technology in therapy against SARS-CoV-2

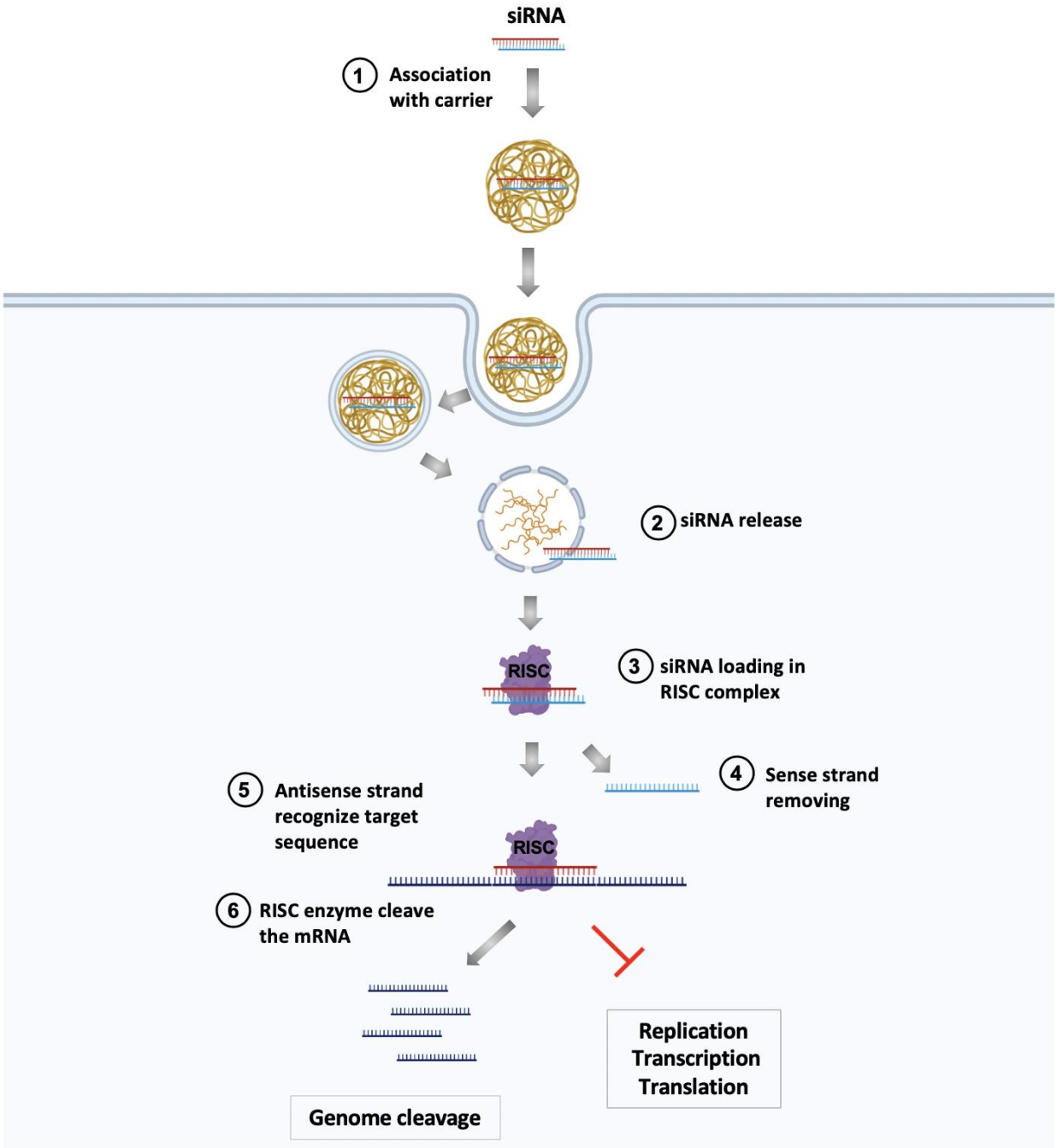
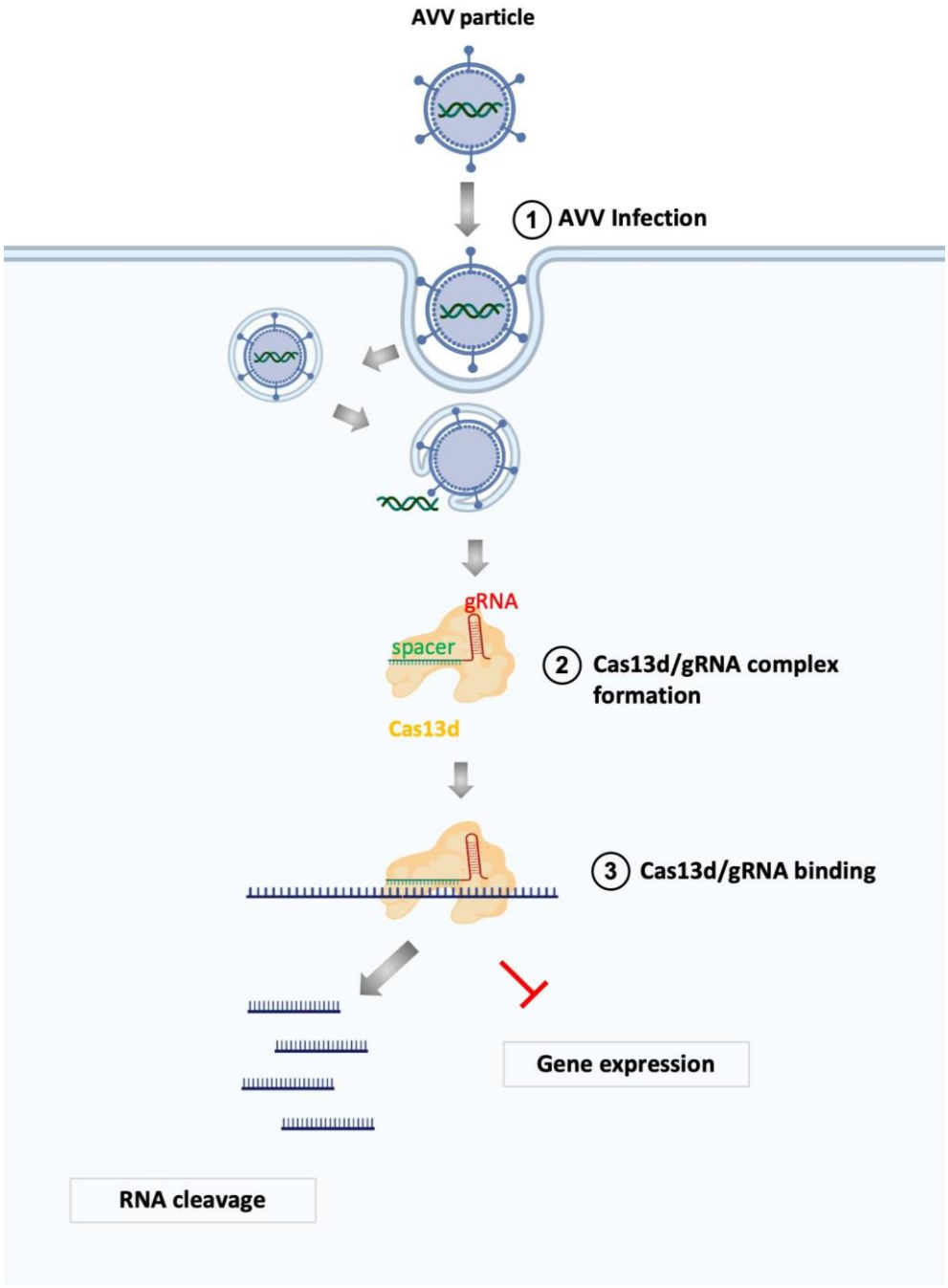


Figure 6. RNA-targeting CRISPR systems for SARS-CoV-2 inhibition



Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: