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Review

Coronavirus RNA Proofreading: Molecular Basis and Therapeutic Targeting

Fran Robson,[1,7] Khadija Shahed Khan,[2,7] Thi Khanh Le,[3,4] Clément Paris,[4] Sinem Demirbag,[5] Peter Barfuss,[6] Palma Rocchi,[4] and Wai-Lung Ng[2,*]

[1]School of Biological Sciences, University of Bristol, Bristol, UK
[2]School of Pharmacy, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong
[3]Life Science Department, University of Science and Technology of Hanoi (USTH), Hanoi, Vietnam
[4]Centre de Recherche en Cancérologie de Marseille, CRCM, Inserm UMR1068, CNRS UMR7258, Aix-Marseille University U105, Institut Paoli-Calmettes, Marseille, France
[5]Faculty of Engineering and Natural Sciences, Sabanci University, İstanbul, Turkey
[6]Université Paris-Est, Cermics (ENPC), INRIA, 77455 Marne-la-Vallée, France
[7]These authors contributed equally
*Correspondence: billyng@cuhk.edu.hk
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SUMMARY

The coronavirus disease 2019 (COVID-19) that is wreaking havoc on worldwide public health and economies has heightened awareness about the lack of effective antiviral treatments for human coronaviruses (CoVs). Many current antivirals, notably nucleoside analogs (NAs), 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 analogs and novel genomic techniques as potential anti-CoV therapeutics that could be used individually or in combination to target the proofreading mechanism.

INTRODUCTION

Coronaviruses (CoVs) are a group of related viruses that belong to the *Coronaviridae* family in the order *Nidovirales* (King et al., 2012). They cause respiratory tract and gastrointestinal tract diseases of varying severity in mammals including humans and birds (Chan et al., 2015; Estola, 1970; Hui and Zumla, 2019; Kahn and McIntosh, 2005; Lai et al., 2020). There are four recognized genera in the subfamily *Orthocoronavirinae*; *Alpha-, Beta-, Gamma-*, and *Deltacoronaviruses*. *Alpha-* and *Betacoronaviruses* infect mammals and include the human 229E and NL63 viruses (*Alpha-*) and OC43 and HKU1 viruses (*Beta-*) that cause common colds (Kahn and McIntosh, 2005). *Gamma-* and *Deltacoronaviruses* mainly affect birds (Woo et al., 2012).

CoVs are enveloped, single-stranded positive-sense RNA viruses with some of the largest known RNA viral genomes, ranging from approximately 26 to 32 kb (Snijder et al., 2003). The surface of each virion is decorated with characteristic club-shaped glycoprotein spikes that bind to host receptors and confer specificity and infectivity (Bosch et al., 2003). These spikes, when the spherical virion particles are viewed under an electron microscope, resemble the solar corona from which they get their name (Almeida et al., 1968).

The current 2019/2020 COVID-19 (coronavirus disease 2019) pandemic is caused by SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2). SARS-CoV-2 is one strain of the severe acute respiratory syndrome-related coronavirus species (SARSr-CoV) in the genus *Betacoronavirus* (Coronaviridae Study Group of the International Committee on Taxonomy of Viruses, 2020). The SARSr-CoV strains include SARS-CoV that was responsible for the SARS outbreak in 2002 and numerous other strains that cause diseases in bats and certain other mammals (Cui et al., 2019; Wong et al., 2019). Another related *Betacoronavirus* member, MERS-CoV, caused the outbreak of Middle East respiratory syndrome disease in 2012 (Chan et al., 2015).

The current knowledge of CoV molecular biology is based on the studies of the *Betacoronavirus* genus including the well-characterized 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 (Wong et al., 2020). Studies of the latter since their emergence in 2003 have generated a vast body of literature, and this will be the major focus of this review (Chan et al., 2015; Compton et al., 1993; Luk et al., 2019).

The Coronavirus Genome

Genome Organization

The polycistronic RNA genome of SARSr-CoV is approximately 30 kb and encodes 14 open reading frames (ORFs), some of which overlap (Snijder et al., 2003). It is capped at the 5′ end and has a 3′ poly(A) tail and short 5′ and 3′ UTR sequences that form regulatory stem-loop structures and a

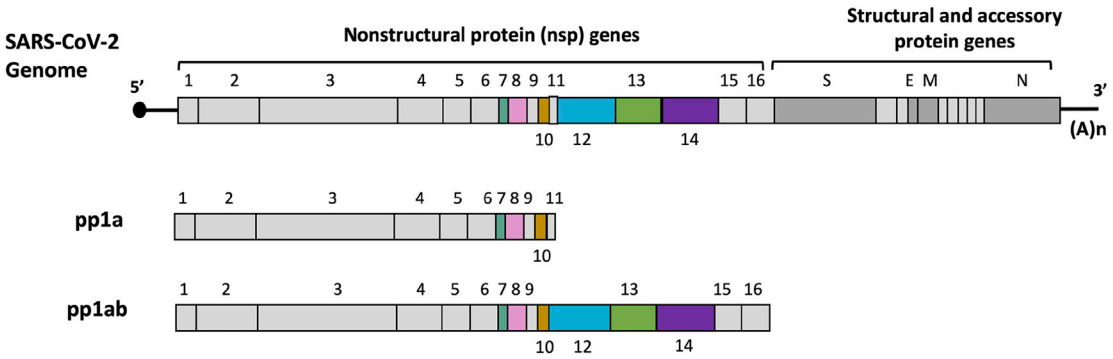


Figure 1. Single-Stranded RNA Genome of SARS-CoV-2
Two-thirds of the genome encodes two large polyproteins, pp1a and pp1ab, that are cleaved into 16 non-structural proteins. The last one-third of the genome encodes structural and accessory proteins. This figure was created with BioRender.

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 ORFs: 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 (Dos Ramos et al., 2004). They are cleaved into 16 non-structural proteins (NSPs) by ORF1a-encoded proteases nsp5 (chymotrypsin-like protease, $3CL^{pro}$, also called main protease or M^{pro}) and papain-like protease (PL^{pro}) residing in subunit nsp3 (Angelini et al., 2013; Snijder et al., 2003). 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 (Table 1). 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) and 6 (SARS-CoV-2) accessory genes respectively with most variation between the two strains found in ORFs 3 and 8 (Malik, 2020).

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. The replication is mediated by the viral RNA-dependent RNA polymerase (RdRp) in non-structural protein 12 (nsp12), along with other replicative enzymes encoded by ORF1b that form replication-transcription complex (Subissi et al., 2014a). 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 (Sola et al., 2011). 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 (Sola et al., 2015).

In a process unique to certain RNA viruses in the order *Nidovirales*, the *Coronaviridae* and *Arteriviridae* families (de Vries et al., 1997), 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 (Sawicki and Sawicki, 1995; Sethna et al., 1989). 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 (Sola et al., 2015). 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 defense mechanisms (Huang et al., 2011). 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.

The Replication-Transcription Complex

CoV 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 (Snijder et al., 2020). Anchorage of RTC to the membranes is mediated by the transmembrane domain of nsp3 together with transmembrane proteins nsp4 and nsp6 (Angelini et al., 2013). 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 (Sola et al., 2015) (Figure 3). The CoV

Table 1. Summary of the Coronavirus Nonstructural Proteins

Function in Virus Life Cycle	Viral Protein Involved	References
Inate host response	Nsp1: cellular mRNA degradation and preventing host mRNA translation through ribosome binding	Cottam et al., 2011; Narayanan et al., 2015; Thoms et al., 2020
	Nsp2: proposed to disrupt host intracellular signaling	
	Nsp6: implicated in the generation of autophagosomes for the degradation of immunomodulatory proteins	
Vesicle membrane formation	Nsp3, nsp4, nsp6: the formation of double membrane vesicles for RNA replication in cytoplasm	Angelini et al., 2013
Cleavage of polyproteins	Nsp3: PL^{pro} domain	Angelini et al., 2013; Snijder et al., 2003
	Nsp5: $3CL^{pro}$ domain	
Replication and transcription	Nsp9: homodimer proposed to function as a single stranded RNA binding protein	Bouvet et al., 2012, 2014; Egloff et al., 2004; Ferron et al., 2018; Imbert et al., 2006; Minskaia et al., 2006; Seybert et al., 2000; te Velthuis et al., 2010
	Nsp13: helicase that unwinds the double strand RNA for nsp12 polymerase	
	Nsp12: RNA-dependent RNA polymerase	
	Nsp8: primase that synthesizes short oligonucleotides to be extended by Nsp12-RdRp; forms a complex with nsp7	
	Nsp14: exonuclease (ExoN) domain that proofreads the nascent RNA strand and excises the misincorporated nucleotides	
	Nsp10: cofactor of nsp14	
RNA capping	Nsp13: RTPase activity that hydrolyses the 5′ end of the RNA strand	Bouvet et al., 2014; Chen et al., 2009; Decroly et al., 2008; Seybert et al., 2000
	Nsp14: N7-methyltransferase, which methylates the guanylate cap	
	Nsp16: 2′-O-methyltransferase methylates the 2′-O-position of the RNA strand	
	Nsp10: cofactor of nsp16	
Others	Nsp15: endoribonuclease	Bhardwaj et al., 2004; Snijder et al., 2016
	Nsp11: unknown	

nsp13 is a helicase that can unwind dsRNA in a 5′ to 3′ direction with the resulting single-stranded RNAs (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 (Adedeji et al., 2012; Seybert et al., 2000).

The nsp12-nsp7-nsp8 Polymerase Complex

The nsp12 protein has two known functional domains: a nidovirus RdRp-associated nucleotidyltransferase (NiRAN) domain at the N terminus that is unique to nidoviruses and the canonical RdRp at the C terminus (te Velthuis et al., 2010; Xu et al., 2003). The RdRp domain 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 nucleoside triphosphate (NTP) by formation of phosphodiester bonds, hence extending the 3′ end of the nascent RNA chain with the incoming nucleotide (Xu et al., 2003). CoV RdRps interact with the nsp7/nsp8 protein complex that confers processivity to the polymerase (Subissi et al., 2014b). Cryoelectron microscopy (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 (Gao et al., 2020). 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 (Hillen et al., 2020). According to the detailed structural analysis, RdRp complexed with one copy of nsp7 and two of 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-helical extensions in nsp8 project out along the existing RNA duplex and interact with bases far from the polymerase core (Hillen et al., 2020). These protrusions are predicted to form positively charged "sliding poles" that could prevent premature dissociation of the replication machinery from its substrate. A mutation in K58 on the N-terminal extension subdomain of nsp8 was found to be lethal to the virus, highlighting the importance of nsp8 interactions with the RNA backbone and its processive replication function (Hillen et al., 2020; Wang et al., 2020b). In addition to providing insight into the processivity of the RdRp complex that is necessary for replication of the very long genomes of viruses in the *Nidovirales* order, this report also modeled the binding of incoming nucleotides to the NTP site demonstrating that contacts between nsp12 and canonical NTPs are conserved.

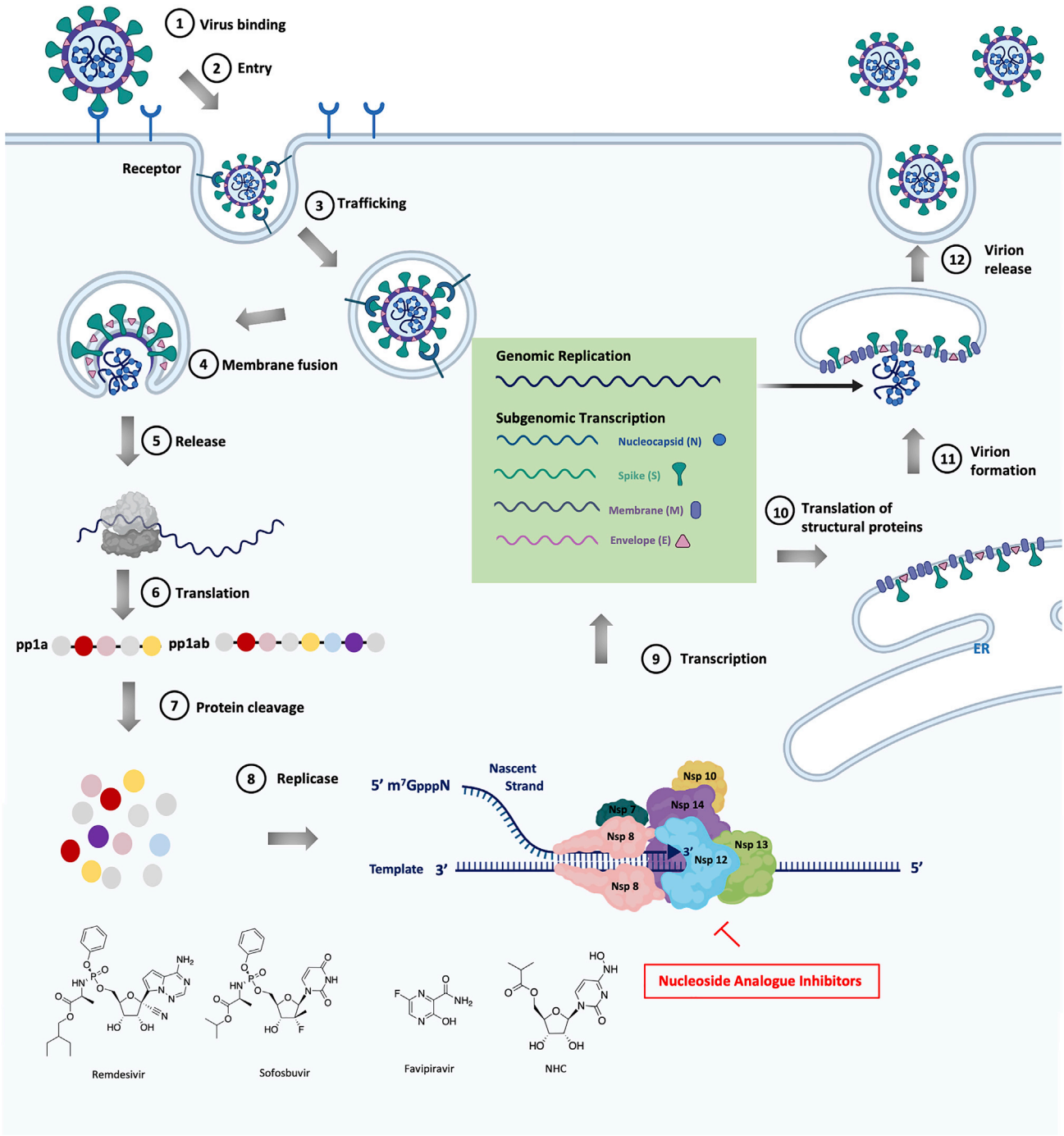


Figure 2. Virus Replication Mechanism

To enter in the host cell, first the virus binds to the ACE2 receptor (1) to initiate the viral entry (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 structural proteins are transcribed (9). Then, the subgenomic mRNAs 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. This figure was created with BioRender.

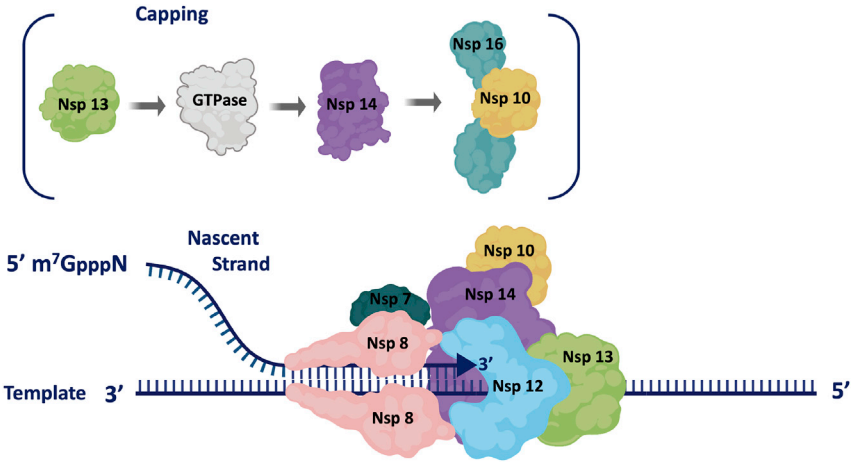


Figure 3. Model of the Core Replication and Proofreading Complex of SARS-CoV
Nsp12-RdRp replicates and transcribes the genome and sgmRNAs. Nsp7/nsp8 proteins confer processivity to the polymerase. Nsp13 unwinds dsRNA ahead of the polymerase. Nsp14-ExoN complexed with its co-factor nsp10 proofreads the nascent RNA strand and excises misincorporated nucleotides. Nsp13, an unknown GTPase, Nsp14-N7-methyltransferase, and the Nsp16-2′-O-methyltransferase/Nsp10 complex are involved in the capping mechanism. This figure was created with BioRender.

mRNA Capping

After replication and transcription, CoV genomic and sgmRNA transcripts are capped at their 5′ end and polyadenylated at their 3′ end by processes presumably similar to those found in eukaryotic cells (Lai and Stohlman, 1981; Peng et al., 2016). 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 recognize uncapped RNAs as "non-self" (Chen and Guo, 2016; Sevajol et al., 2014). Unlike cellular mRNA capping that takes place in the nucleus, viral capping occurs in the cytoplasm and consequently is mediated by nsp13, nsp14, and nsp16 (Chen and Guo, 2016). CoV mRNA cap structures consist of a 7-methylguanosine linked to the first nucleotide of the RNA transcript through a 5′-5′ triphosphate bridge (m^7GpppN), 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" ($m^7Gppp_{m2}N$) (Bouvet et al., 2010; Sevajol et al., 2014; Sola et al., 2015) (Figure 3).

Coronavirus Proofreading Mechanism

RNA virus replication typically has a high error rate (or low viral fidelity) that results in the virus existing as diverse populations of genome mutants or "quasispecies" (Denison et al., 2011). While low replicative fidelity allows the RNA viruses to adapt to different replicative environments and selective pressures, it is also associated with an increased chance of error catastrophe leading to viral extinction. This suggests the need for a finely tuned balance between quasispecies diversity and replicative fitness for viral virulence and evolution (for comprehensive reviews on replication fidelity and diversity, please see Denison et al., 2011; Gorbalenya et al., 2006; Smith and Denison, 2012). This factor in itself challenges development of antivirals against CoVs and other RNA viruses as they can rapidly develop resistance to drugs while maintaining viral replicative fitness (Perales and Domingo, 2016). An additional barrier to the development of NAs as antivirals against CoVs is the unique 3′ to 5′ exonuclease (ExoN) proofreading function that has been demonstrated for some CoV nsp14 enzymes; MHV and SARS-CoV nsp14-ExoN mutants accumulated up to 20-fold more mutations than their wild-type counterparts (Eckerle et al., 2007, 2010). CoV genomes and others in the *Nidovirales* order are the largest and most complex known RNA virus genomes (Saberi et al., 2018), and nsp14 is highly conserved within the *Coronaviridae* family (Ma et al., 2015). 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 (Gorbalenya et al., 2006).

The Bifunctional nsp14 Protein

Nsp14 is a 60 kDa bifunctional enzyme with an N-terminal exonuclease (ExoN) domain implicated in replication fidelity and a C-terminal N7-methyltransferase (N7-MTase) domain involved in mRNA capping (Chen et al., 2009) (Figure 4A). In addition, nsp14 is also involved in several other processes of the virus life cycle and pathogenicity, including innate immune responses and viral genome recombination (Becares et al., 2016; Gribble et al., 2020).

N7-Methyltransferase Domain

The C-terminal N7-MTase domain contains a DxG S-adenosyl-L-methionine (SAM)-binding motif that is conserved among CoVs and is involved in mRNA capping and host immune response evasion (Case et al., 2016). The capping pathway in CoVs is thought to comprise 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-methyltransferase, forming the "cap-0" structure in the presence of methyl donor, S-adenosyl-L-methionine (SAM); (4) methylation of the 2′O-position of the first RNA molecule in the chain by the nsp10/nsp16 SAM-dependent 2′-O-methyltransferase (2′-O-MTase) to form the "cap-1" structure (Chen and Guo, 2016; Decroly et al., 2011; Sevajol et al., 2014). The capped RNA molecules are recognized by the host eukaryotic initiation factor 4E (eIF4E) that subsequently recruits the cellular translational machinery (Decroly et al., 2011). Mutations in the DxG motif in MHV nsp14 (G332A) and SARS-CoV nsp14 (G333A) specifically abolish the N7-MTase activity that is crucial for mRNA cap formation, and they were shown to have a clear detrimental

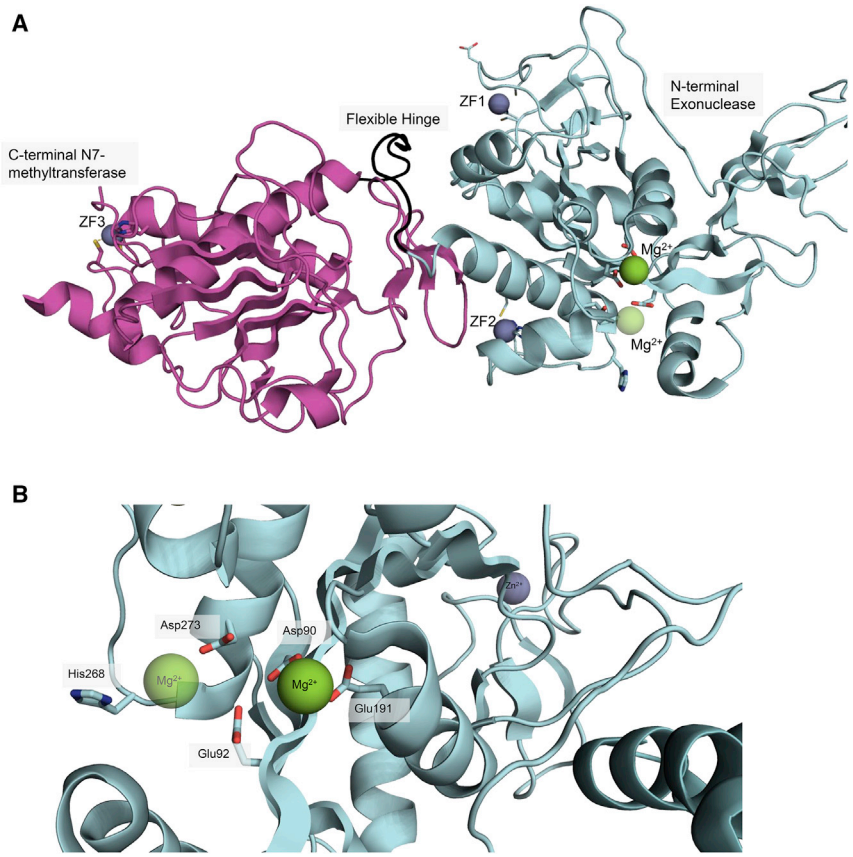


Figure 4. The Overall Structure of Nonstructural Protein 14

(A) Cartoon representation of the structure of the nsp14. The N-terminal exonuclease domain (aquamarine) and C-terminal N7-methyltransferase (light magenta) are connected by a flexible interdomain loop (black). The amino acid residues that coordinate zinc fingers (ZF) (slate blue) and magnesium cofactor (Mg^{2+}) (pea green) are shown as sticks. Protein structure is retrieved from Protein Data Bank (PDB ID: 5C8U; Ma et al., 2015).

(B) Detailed cartoon representation of the catalytic DEEDh residues. DEEDh domain comprises Asp90, Glu92, Glu191, Asp273, and His268 amino acids residues that are located in the exonuclease domain (aquamarine) of the nsp14. Mg^{2+} cofactor (pea green) is coordinated by Asp90 and Glu191 and is thought to facilitate the removal of misincorporated nucleotides. The second Mg^{2+} is shown tentatively; isothermal titration calorimetry predicted a two-metal binding mode for divalent cations, but crystallography data showed only one (Chen et al., 2007). The protein structure was retrieved from Protein Data Bank (PDB: 5C8U; Ma et al., 2015).

effect on replication without disrupting the ExoN activity, demonstrating the importance of this enzymatic function of nsp14 (Case et al., 2016).

Exonuclease Domain

The nsp14 exoribonuclease activity resides in the N terminus of this bifunctional protein (Chen et al., 2009; Denison et al., 2011; Snijder et al., 2003). 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 (Ferron et al., 2018; Ogando et al., 2019). A recent report demonstrated that the ExoN domain in nsp14 plays a role in sgmRNA recombination frequency and recombination patterns in virally infected cells and virions. A viable 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 (DVGs) were also observed in MHV-ExoN(–), further suggesting the importance of ExoN in viral RNA synthesis and viral fitness (Gribble et al., 2020).

DEDDh Superfamily

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 (Snijder et al., 2003). 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 (Beese and Steitz, 1991; Chen et al., 2007).

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 homologs in the DEDD superfamily and cast light on several features of the protein's structure and function (Ma et al., 2015). 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 (Figure 4B); 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 places it in the DEDDh subfamily. Simultaneous mutations in 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.

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 nsp14 (Chen et al., 2007; Minskaia et al., 2006). 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 (Minskaia et al., 2006). While MHV and SARS-CoV 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 (Eckerle et al., 2007, 2010). Recently, Ogando et al. tested equivalent knockout mutants of MERS-CoV ExoN active site and demonstrated that these are non-viable and completely lacking in RNA synthesis. Only one mutant out of 12 tested could be recovered, likely due to the conservative residue change (D191E). The wild type and the D191E mutant showed comparable sensitivity to the mutagen 5-fluorouracil (5-FU) with only a very slight increase in sensitivity of the mutant over WT. N7-MTase activity was found to be normal in the ExoN mutants demonstrating that the lethal effects of the substitutions were not due to inadvertent effects on N7-MTase function. Together, these data reveal an additional and more fundamental role for ExoN in viral RNA synthesis in some CoVs (Ogando et al., 2020).

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+} (coordinated by Asp90 and Glu191) was observed (Figure 4B). This could be explained by the lack of substrate or product binding (Chen et al., 2007; Ma et al., 2015).

The Role of Zinc Finger Motifs in Viral Replication

In addition to the revised catalytic motif, a study revealed significant differences between nsp14 ExoN and other members of the DEDD exonuclease superfamily (Chen et al., 2007). 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 a nsp10 interaction site. 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 in SARS-CoV. However, Ogando et al. demonstrated one viable but severely crippled zinc finger 1 mutation, H229C, where the amino acid substitution preserves the zinc-coordinating properties in MERS-CoV. It was proposed that the close proximity of zinc finger 1 to the nsp10 interaction site (discussed below) might affect the interaction between nsp10 and nsp14 and thereby reduce the ExoN catalytic activity. Ogando et al. also found that mutations in zinc finger 2 completely abolished the viral viability, which is consistent with the previous findings (Ogando et al., 2020). The induction of a specific mutation in 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 (Becares et al., 2016).

The Interaction between nsp14-ExoN and the nsp10 Cofactor

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 (Pan et al., 2008). *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, suggesting a role for nsp10 in stabilizing the ExoN active site in the correct conformation for substrate catalysis (Bouvet et al., 2012, 2014).

The nsp10 interacts with N-terminal Ala1-Arg76 and a β-hairpin structure containing β5 and β6 Ala119-Asp145 of the nsp14, revealing the complex structure of a one-to-one ratio of nsp10 to nsp14 molecules (Ferron et al., 2018; Ma et al., 2015). The interaction of nsp10 with nsp14 is like a hand (nsp14) over a fist (nsp10). 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 (Bouvet et al., 2014; Ma et al., 2015). Small-angle X-ray scattering (SAXS) analysis shows, in the absence of nsp10, there is a drastic deformation of the nsp14 exonuclease site, suggesting that nsp10 stabilizes the catalytic site of ExoN and renders it in an effective active conformation (Ferron et al., 2018).

The Interaction between nsp14 and the RdRp Complex

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 (Subissi et al., 2014b). Another study that involved SAXS and mutagenesis of key residues showed 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 (Ferron et al., 2018).

In sum, the bifunctional Nsp14 protein clearly plays a number of important roles in viral replication. The high conservation of the ExoN sequences among *Coronaviridae* family members and the lack of close orthologs in the host genome suggest that the ExoN domain could be a potential target for the development of novel antiviral drugs. In the next section, we consider antiviral strategies that could evade the CoV proofreading ability or target ExoN to improve drug potency.

Potential Anti-CoV Strategies Targeting Replication Fidelity

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. To date, remdesivir, an investigational nucleoside analog, is the only antiviral that has been granted emergency use status for the treatment of SARS-CoV-2 (Wang et al., 2020c). In this section, we discuss specific antiviral compounds and therapeutic approaches that could target CoV replication fidelity and evade the proofreading machinery.

Nucleoside Analogs

Nucleoside analogs (NAs) are synthetic derivatives of natural nucleosides, with a long history of use as anticancer, antiviral, antibacterial, and immunomodulator drugs (Seley-Radtke and Yates, 2018). Since the first successful clinical use in the 1960s of an NA, 5-iododeoxyuridine, for the treatment of herpes virus infections, NAs are now the largest class of the small-molecule antiviral therapeutics (Seley-Radtke and Yates, 2018). They are designed to mimic naturally occurring nucleosides and be recognized by the viral and cellular transcriptional machinery. However, once they are incorporated into RNA or DNA strands in their active form, NAs can exert their inhibitory effect in two ways: (1) they can mispair with natural nucleotides leading to the assimilation of errors in the RNA strand that result in lethal mutagenesis; and (2) their incorporation may prematurely terminate the growing RNA or DNA chains. Obligate chain terminator NAs lack the ribose-3′-OH moiety, which is required for polymerization of the next NTP and once incorporated, further RNA synthesis is inhibited immediately (Deval, 2009). Non-obligate chain terminators 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 NA moves through the protein complex thus halting synthesis. In this case, chain termination may be immediate or may occur following a limited extent of continued RNA or DNA synthesis (delayed) (De Clercq and Neyts, 2009). In addition to these two mechanisms of action, the pool of naturally occurring cellular nucleotides may be depleted or biased by the presence of NAs (Pruijssers and Denison, 2019).

Ideally, a successful NA-based antiviral therapy should selectively target the viral genome, be effective at low micro molar concentrations to minimize cellular toxicity (a low micro molar IC_{50} value), and demonstrate a high barrier to resistance. Antiviral nucleoside analogs 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 (Feng, 2018). On the other hand, the structural conservation of the polymerase binding sites targeted by NAs is high among virus families and therefore mutations that confer NA resistance often result in diminished virus fitness. In this respect, NAs are good antiviral candidates as they demonstrate a relatively high barrier to resistance emergence (Jordan et al., 2018). There are now several examples of successful clinical therapies using NAs to target a range of human and domesticated animal viruses (Seley-Radtke and Yates, 2018); for example, the NAs entecavir and telbivudine against hepatitis B viruses (HBVs) (Liu et al., 2014) and the metabolized form of RDV; GS-441524; for treatment of the causative agent of feline infectious peritonitis (FIPV) (Pedersen et al., 2019).

Repurposing Existing Antiviral Nucleoside Analogs

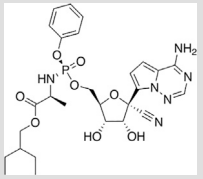
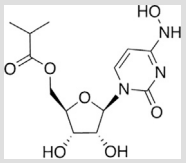
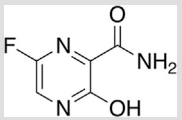
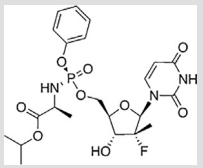
Repurposing existing antiviral drugs, both approved and experimental, to fight CoVs has been widely recognized as the quickest strategy to obtain effective therapies to control CoV outbreaks (Li and De Clercq, 2020). However, the unique ability of CoVs to remove mismatched nucleotides during RNA synthesis has resulted in native resistance of CoVs to many NAs including some that have proven antiviral properties but whose success relies on viral replication infidelity (Eckerle et al., 2007; Ferron et al., 2018; Smith et al., 2013). Nevertheless, there are a number of NAs that are being examined for anti-CoV activity *in vitro* and have shown promising results. We review a selection of key examples here and summarize in Table 2.

Remdesivir

Remdesivir (RDV; GS-5734), an experimental drug, was developed by Gilead Sciences to treat Ebola and related viruses. GS-5734 is a broad spectrum prodrug that is metabolized into its active parent nucleoside, GS-441524, an adenosine nucleotide analog (Warren et al., 2016). The active compound targets the viral RdRp enzyme and interrupts RNA genome synthesis (Warren et al., 2016). *In vitro* and animal studies demonstrated antiviral activity of RDV against CoVs including SARS-CoV and MERS-CoV (Agostini et al., 2018; Sheahan et al., 2017, 2020a; de Wit et al., 2020) and SARS-CoV-2 (Wang et al., 2020a). Lo et al. also demonstrated that the combination of remdesivir and the HCV protease inhibitor simeprevir synergistically suppresses SARS-CoV-2 replication *in vitro* (Lo et al., 2020).

RDV exerts its effect first by competing with ATP for binding to RdRp and then attenuating replication by delayed chain termination. Using *in vitro*-expressed active complexes of the SARS-CoV-2 RdRp with cofactor nsp8, Gordon et al. demonstrated that the active form of RDV displays high selectivity over its natural counterpart, ATP (Gordon et al., 2020). In addition, the chain termination mechanism of RDV is not "classic" but results in termination after a further incorporation of up to three nucleotides at position i+3 (Gordon et al., 2020). RDV has a normal ribose 3′-OH that presumably allows

Table 2. Nucleoside Analogs Showing Promise against Coronaviruses

Nucleoside Analog	Structure[a]	Proposed Mode of Action	Approved Indication	Antiviral Potency IC_{50} value (μM)	Current Status[b]	Reference
Remdesivir (GS-5734)		competition with ATP for RdRp binding non-obligate delayed chain termination some resistance to excision by ExoN	experimental drug for Ebola infection	MERS-CoV: 0.074 (measured viral gene copy in infected HAE) SARS-CoV: 0.069 (measured viral gene copy in infected HAE) SARS-CoV-2: 4.269 (measured viral gene copy in infected Vero E6)	FDA emergency use authorization for COVID-19	Agostini et al., 2018; Lo et al., 2020
β-D-N4-hydroxycytidine/ NHC / EIDD-1931 (Prodrug: EIDD-2801)		lethal mutagenesis resistance to excision by ExoN alteration of RNA secondary structure blockage of virion release	experimental drug for various viruses, including VEEV and human coronavirus NL63	MERS-CoV: 0.024 (measured viral gene copy in infected HAE) SARS-CoV: 0.14 (measured viral gene copy in infected HAE) SARS-CoV-2: 0.30 (measured viral gene copy in infected Vero E6)	recruiting participants for clinical trials	Sheahan et al., 2020b
Favipiravir (T-705)		non-obligate delayed chain termination lethal mutagenesis biased cellular nucleotide pool slowed RNA synthesis	approved drug for influenza infection	SARS-CoV-2: 61.88 (measured viral gene copy in infected Vero E6)	two ongoing clinical trials	Wang et al., 2020b
Sofosbuvir		non-obligate chain termination	approved drug for Hepatitis C virus	high binding energy to SARS-CoV-2 nsp12/nsp7/nsp8 complex. *In vitro* data using infected Vero E6 cells showed that Sofosbuvir failed to exert antiviral activity against SARS-CoV-2	recruiting participants for clinical trials	Liu et al., 2020; Elfiky 2020

HAE, human airway epithelial cell; VEEV, Venezuelan equine encephalitis virus; MERS-CoV, Middle East respiratory syndrome coronavirus; SARS-CoV, severe acute respiratory syndrome coronavirus.

[a]Prodrug structures are shown.

[b]Clinical trials (ClinicalTrials.gov) and FDA status (FDA.gov): at date of submission of manuscript.

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 MHV lacking nsp14 ExoN proofreading was significantly more sensitive *in vitro* to RDV than the WT virus, demonstrating that ExoN in addition to RdRp plays a role in virus resistance to this drug (Agostini et al., 2018; Eckerle et al., 2007).

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 (Shannon et al., 2020a). They then mapped the active metabolite 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 occur at the active site of ExoN.

Several articles have reported 3D structures of the SARS-CoV-2 RdRp protein, alone or complexed with partner proteins, RNA substrates, and inhibitors (Elfiky, 2020; Gao et al., 2020; Gordon et al., 2020; Hillen et al., 2020; Shannon et al., 2020a; Yin et al., 2020). *In silico* molecular modeling studies using the published sequence of SARS-CoV-2 RdRp and available resolved structures of SARS-CoV (Elfiky, 2020; Shannon et al., 2020a) and *in vitro* cryo-EM of SARS-CoV-2 RdRp (Gordon et al., 2020; Wang et al., 2020b; Yin et al., 2020) sought to map promising antiviral compounds including remdesivir and its active metabolite to the active site of RdRp. Polymerase assays using SARS-CoV-2 RdRp complexed with nsp7-nsp8 and bound to a template-primer RNA molecule demonstrated inhibition upon the addition of RDV-TP (Yin et al., 2020). Thus far, RDV, an antiviral originally designed against Ebola, fits the criteria for an anti-CoV therapeutic: selective incorporation into RNA strands by RdRp and some resistance to excision by ExoN. RDV has now been granted emergency use status for the treatment of SARS-CoV-2 (Wang et al., 2020c).

β-D-N4-Hydroxycytidine

β-D-N4-hydroxycytidine (NHC; EIDD-1931), a cytidine analog with a modified nucleobase, has antiviral activity against a broad spectrum of viruses including coronaviruses such as HCoV-NL63 and SARS-CoV (Barnard et al., 2004; Pyrc et al., 2006). An *in vitro* study demonstrated that NHC effectively inhibits two different β-CoVs; MHV and MERS-CoV with only slight toxicity and low frequency of resistance (Agostini et al., 2019). NHC antiviral activity is likely in part due to lethal mutagenesis of the viral genome (Pruijssers and Denison, 2019), although the detailed mechanisms of NHC antiviral activities are yet to be discovered. NHC is the first mutagenic NA 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 (Agostini et al., 2018). One possible explanation for this observation is that NHC is not recognized, 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 had suggested that NHC might alter RNA secondary structure or block the virion release process (Stuyver et al., 2003; Urakova et al., 2018). Of note, it has recently been reported that NHC effectively targeted different zoonotic CoVs including SARS-CoV-2 in both *in vitro* and animal models, with an enhanced efficiency against coronavirus containing resistance mutations to RDV (Sheahan et al., 2020b).

Favipiravir

Favipiravir (T-705; 6-fluoro-3-hydroxy-2-pyrazinecarboxamide) was first developed as an anti-influenza virus therapy targeting the viral RdRp and has subsequently demonstrated broad spectrum antiviral activity (Furuta et al., 2002, 2013, 2017). Favipiravir is a purine nucleoside analog 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, human hypoxanthine guanine phosphoribosyltransferase (HGPRT); the active triphosphate then disrupts influenza viral RNA replication by incorporating into the growing RNA chain (Naesens et al., 2013). There were two proposed mechanisms of antiviral action of T-705-RTP against the seasonal influenza virus H1N1, the first via non-obligate chain termination (Jin et al., 2013; Sangawa et al., 2013) and the second via lethal mutagenesis (Baranovich et al., 2013). It was also hypothesized that the increased mutation rates observed in response to T-705 treatment could 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 rationalized the apparent contradiction between the chain termination and lethal mutagenesis theories (Shiraki and Daikoku, 2020). However, in a more recent study favipiravir was found to be exhibiting its antiviral activity against SARS-CoV-2 via a combination of mechanisms, including slowing RNA synthesis, initiating chain termination, and inducing lethal mutagenesis (Driouich et al., 2020; Shannon et al., 2020b), demonstrating that these mechanisms are not mutually exclusive. In addition, genomic analysis revealed resistance mutations against favipiravir were acquired in nsp14 proofreading coding region although the impact of these mutations is yet to be investigated (Driouich et al., 2020).

Sofosbuvir

Sofosbuvir is a broad-spectrum NA licensed for the treatment of hepatitis C virus (HCV) infection. The prodrug is metabolized 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 that can bind to the RdRp active domain, incorporate into the nascent RNA strands and block

chain extension (Appleby et al., 2015; Noell et al., 2015). This is likely due to the presence of the 2′F-2′C-Me group in the triphosphate form of sofosbuvir that leads to steric clashes with the incoming NTP and termination of HCV RNA synthesis (Fung et al., 2014). Sofosbuvir is one of several antiviral compounds that is predicted to bind tightly to SARS-CoV-2 RdRp in molecular modeling experiments (Elfiky, 2020). And in homology and structural superimposition studies the residues in the HCV RdRp that bind sofosbuvir were found to be conserved in several SARS-CoV-2, SARS, and MERS-related CoV polymerases (Jácome et al., 2020). A study using polymerase extension assays with a pre-annealed RNA template and primer and a pre-assembled SARS-CoV-2 nsp12-nsp8-nsp7 complex demonstrated that sofosbuvir could block chain extension (Chien et al., 2020). However, a study that evaluated 19 potential anti-CoV drugs demonstrated that sofosbuvir failed to protect cells against SARS-CoV-2-induced cytopathic effects (Liu et al., 2020). Further studies *in vitro*, in animal models, and in clinical trials are needed to establish whether this, or any drug under investigation, will be effective against CoVs.

Limitations of Nucleoside Analogs

There are a number of limitations associated with NAs as drugs; they cannot be administered directly in the active triphosphate form as these molecules are highly charged and not cell permeable. Thus, NAs are usually given as a prodrug that, once it enters the cell, must be phosphorylated into the triphosphate form to be recognized by the polymerase (De Clercq and Neyts, 2009; Deval, 2009; Yates and Seley-Radtke, 2019). The first phosphorylation reaction to convert NA into the monophosphate form is often very specific and can be rate limiting; thus, the NA may not be recognized and seems inactive (Mehellou et al., 2018). To overcome these impediments, during the development of remdesivir, the 1′-CN parent nucleoside GS-441524 was modified using the McGuigan ProTide strategy (McGuigan et al., 1996; Mehellou et al., 2018). The monophosphate prodrug remdesivir carries ethylbutyl ester and phosphoramidate groups that mask the negative charge, thus allowing it to cross the cell membrane; this also bypasses the rate-limiting first phosphorylation step (Siegel et al., 2017). There are also many examples of NA delivery strategies to improve absorption of prodrugs in a form that can be more efficiently metabolized. Due to space limitations, we cannot discuss them fully here, but we direct readers to the following reviews (Diab et al., 2007; Jordheim et al., 2013; Zhang et al., 2014).

It should be noted that although ribavirin (RBV), a broad-spectrum antiviral NA, has been used clinically to manage CoV infections (Hung et al., 2020), *in vitro* experiments using isolates of SARS-CoV suggested that ribavirin was excised from the viral genome due to the proofreading activity of the nsp14 ExoN domain (Ferron et al., 2018). Thus, this represents a potential mechanism of resistance to some NAs. In another *in vitro* study while the WT SARS-CoV was resistant to both RBV and the mutagen 5-FU, knocking out ExoN function resulted in 300-fold greater sensitivity to the compounds, demonstrating the importance of exonuclease activity in CoV resistance to NAs (Smith et al., 2013). Rational design of NAs, which could evade the ExoN proofreading activity, is therefore highly warranted.

Of note, although the primary consequence of the inhibition of ExoN proofreading activity is lethal mutagenesis, selective targeting of the viral proofreading machinery could potentially create an undesired selection pressure, thereby leading to the emergence of a small number of even more viable mutated strains. To overcome some of the challenges of NAs as therapeutic drugs against CoVs, namely, delivery to the lungs and into the cells, undesired selection pressure, and issues with potency that might arise from CoV proofreading function, we next consider combination therapies of NAs with novel genomics technologies.

Antisense Oligonucleotides

Antisense oligonucleotides (ASOs) represent a promising class of compounds that can be developed quickly and specifically against CoVs. ASOs are single DNA strands of around 20 nucleotides that specifically hybridize to the complementary sequence of the target RNA. Depending on the type of ASO, the resulting DNA:RNA hybrid can 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 (Karaki et al., 2019). To date, there are seven FDA-approved ASO therapies, including Vitraven (cytomegalovirus retinitis, 1998) and Exondys 51 (Duchenne muscular dystrophy, 2016) (Stein and Castanotto, 2017).

A number of antiviral ASOs have been successfully designed to inhibit various influenza viruses (Duan et al., 2008; Gabriel et al., 2008; Kumar et al., 2013). A modified ASO, phosphorodiamidate morpholino oligomer (PMO), was explored in the treatment of Ebola virus, flaviviruses, coronaviruses, and picornaviruses (Nan and Zhang, 2018). PMOs primarily exert antiviral activity in RNase H-independent manner by base-pairing to the target RNA and sterically inhibiting RNA processing, subsequently shutting down RNA translation or potentially viral RNA replication (Nan and Zhang, 2018). PMOs are frequently designed to bind to the translation start site (AUG) region, the 3′-terminal region, or transcription regulatory sequences (TRS) of mRNA and RNA genome segments. The antiviral potency of PMO targeting the 5′ end of the MHV genome was demonstrated by interrupting the translation process of the viral replicase polyprotein. These ASOs were shown to effectively enter host cells and decrease CoV growth and proliferation (Neuman et al., 2004).

Rationale of Using ASOs against CoVs

The high mutation rate of CoVs could potentially complicate the discovery of conventional antiviral drugs that are usually time consuming to develop and clinically evaluate. A genomic study analyzed SARS-CoV-2 sequences from patients in three different countries (USA, China, Australia) and found several single-nucleotide polymorphisms (SNPs) 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 impact the current treatment strategies and allow the virus to develop antiviral resistance (Nguyen et al., 2020). ASO-based

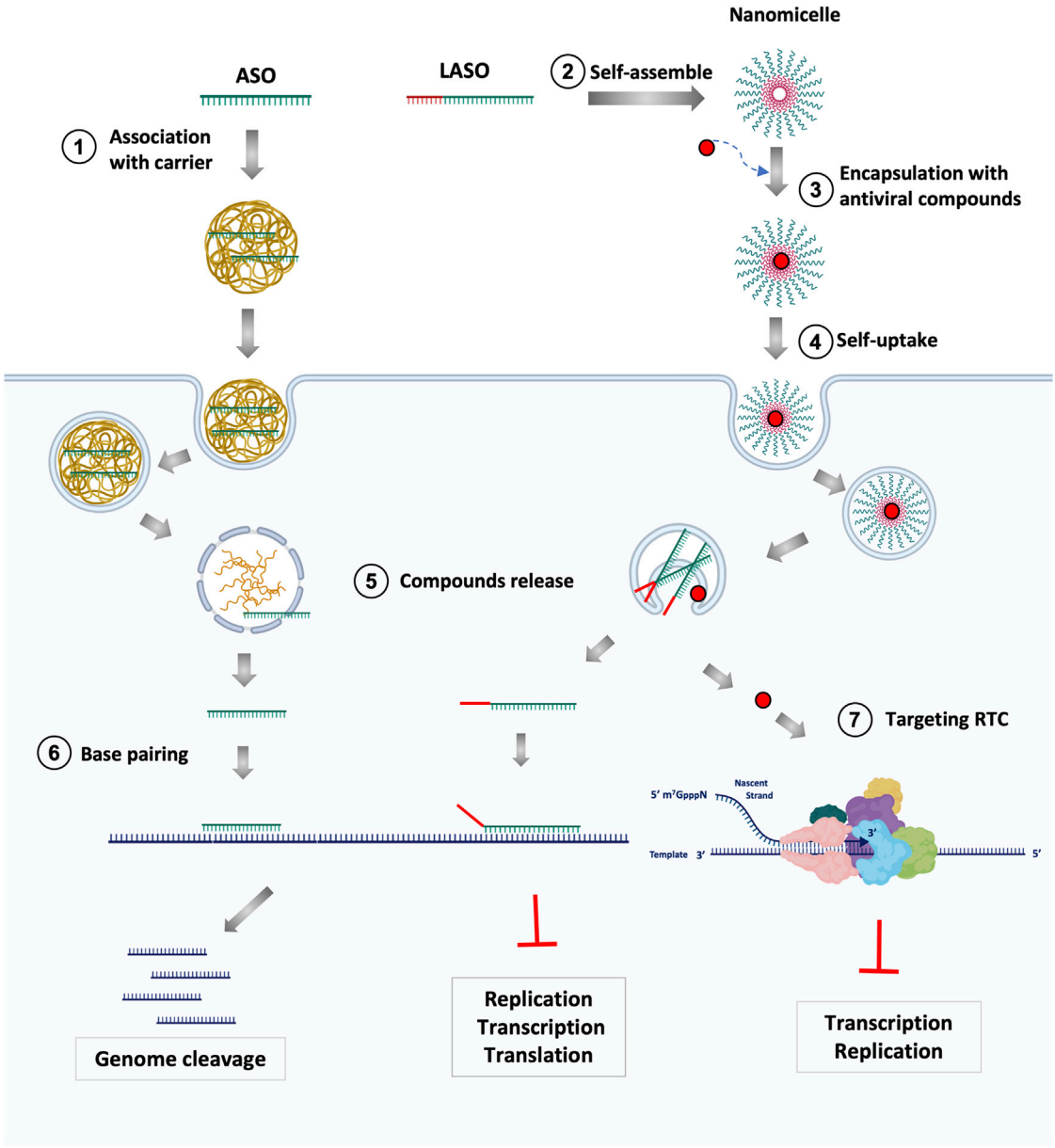


Figure 5. ASO Technology in Therapy against SARS-CoV-2

Antisense oligonucleotides (ASOs) are conjugated with a carrier that allows delivery into the cells (1). Lipid-modified ASOs (LASO) self-assemble into nanomicelles (2) and encapsulate an antiviral molecule such as an NA (3). These nanomicelles are able to enter the cell without any transfection agents (4). Once inside the cell, ASO, LASO, and the encapsulated drugs are released (5). ASOs are administered via cationic polymers and are released through the proton sponge effect (the presence of the weakly basic molecule causes the endosome to burst). LASOs enter via the macropinocytosis mechanism and are released through interaction with the endosomal membrane. The intracellular ASOs/LASOs match to their complementary sequences (6), leading either to genome degradation (through RNaseH activity) or replication/transcription/translation blocks due to steric block formation. The NAs can interfere with the RNA replication and transcription by targeting RdRp as described above (7). This figure was created with BioRender.

approaches might be worth exploring due to the high specificity, affinity, and relatively simple rational design of oligonucleotides. Notably, due to their resistance to various enzymes including nucleases, ASOs can be stable for hours in both cellular and extracellular environments, making ASOs highly applicable to *in vivo* models and clinical applications (Nan and Zhang, 2018; Summerton, 1999). ASOs are generally administered by subcutaneous injections but can also be administered by aerosol inhalation (Crosby et al., 2017). A combined therapy comprising ASOs and NAs could be explored and the antiviral activity of these drugs could potentially be enhanced.

Novel ASO-Based Strategies against CoVs

The antiviral effect of morpholino-type ASOs specifically targeting the TRS located in the 5′ UTR of SARS-CoV was demonstrated by the efficient blockage of genome replication (Neuman et al., 2005). Using ASOs to target and cleave any regions of the long CoV genome transcript that encodes the polyprotein could result in complete shutdown of viral transcription as this strategy could effectively abolish all NSP protein expression. Designing the ASO to highly conserved regions of the CoV genome such as the sequence encoding RdRp or the nucleocapsid mRNA could result in highly specific ASOs and may reduce the probability of resistance mutations arising (Abbott et al., 2020). An alternative strategy would involve a combination therapy. ASOs could be designed to target structural protein genes only. These ASOs could be administered alongside NAs that target the RTC so that two crucial processes in the viral life cycle are targeted at the same time. To improve the potency of NAs a further strategy that specifically knocks down ExoN expression could be considered; as the NSPs are translated as a polyprotein knocking out a single gene without affecting other NSPs is tricky. However, ASOs can be used to alter splicing and to skip exons (Shimo et al., 2018) so could potentially be designed to selectively disrupt ExoN. A high-resolution map of SARS-CoV-2 transcriptome has revealed considerable complexity including alternative splicing events that introduce fusions, deletions, and frameshifts (Kim et al., 2020). Identifying the non-canonical RNA modification sites could aid the design of ASOs to splice out ExoN and other specific targets.

Recently, Rocchi et al. demonstrated that lipid-modified ASOs (LASOs) have the ability to self-assemble into ''nanomicelles,'' enabling efficient cellular uptake of ASO without the need for a transfecting agent (Karaki et al., 2017; Rocchi et al., 2013a, 2013b). Furthermore, these nano-structures can encapsulate hydrophobic chemotherapeutics and assist their delivery (Karaki et al., 2018). They demonstrated that chemotherapy encapsulation in LASO nanocomposites allows a greater solubility of the compound in water and greatly increases the efficacy of the compounds. As the NA remdesivir, for example, is poorly soluble in water and requires betadex sulfobutyl as a solubilizing agent, the LASO technology holds promise as a drug delivery method of this compound to enhance the effective concentration in the targeted organ. Importantly, the physicochemical properties of formed hybrid nanomicelles allow them to be administered by inhalation of an aerosol, which could significantly reduce toxicity and target the antiviral compound to the lungs. Thus, encapsulation of NAs such as RDV in antiviral LASO nanocomposites could function as a combinatorial therapy to simultaneously deliver specific ASOs along with NAs and to enhance the potency of NAs or other antiviral treatments (Figure 5).

Conclusions and Future Directions

The 21st century has seen several outbreaks of serious zoonotic CoV diseases: SARS-CoV in 2002/3, the ongoing MERS-CoV that started in 2012, and the current SARS-CoV-2 worldwide 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 CoV 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 CoVs. 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 NAs could enhance the effectiveness of the NAs 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.

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AUTHOR CONTRIBUTIONS

F.R., K.S.K., and W.-L.N. determined the scope of this review. All authors were involved in the writing and editing of the manuscript. C.P., K.S.K., T.K.L., and S.D. prepared the figures.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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