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REVIEW

Targeting coronaviral nsp14: a bifunctional enzyme for RNA capping and proofreading

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Abstract Over the past thirty years, coronaviruses (CoVs) have caused several pandemics, posing significant public health and economic burdens. Although the development of vaccines and the emergence of antiviral therapeutics have played a crucial role in mitigating pandemics caused by CoVs, there is an urgent need for alternative strategies to combat potential future pandemics. CoVs possess the largest genomes of all known RNA viruses, and they employ a proofreading mechanism mediated by nsp14 ExoN to maintain the fidelity of their genomes. Additionally, the N7-MTase of nsp14 can catalyze the formation of a cap(0) structure (^{7Me}GpppA) that allows the virus to evade 5'-3' ExoN-mediated hydrolysis in host cells. Therefore, as a bifunctional enzyme, nsp14 is a promising potential target for inhibiting viral proliferation. Here, we review the functions of CoV nsp14 and their underlying molecular basis, highlighting the potential and importance of nsp14 as a drug target. We also describe the existing small molecules known to interfere with the ExoN or MTase activity of nsp14, providing inspiration for the development of anti-CoV agents.

Keywords Coronavirus, Nsp14, Proofreading, Capping, Inhibitors

INTRODUCTION

Coronaviruses (CoVs), a family of enveloped, positive-sense single-stranded RNA viruses, have emerged as formidable threats to global health. Over the past three decades, several pandemics have been caused by CoVs, such as severe acute respiratory syndrome coronavirus (SARS-CoV) (Drosten *et al.* 2003), Middle East respiratory syndrome coronavirus (MERS-CoV) (The Lancet 2013) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Zhu *et al.* 2020), all of which belong to the β-coronavirus genus. CoVs possess the largest genome among RNA viruses, at approximately 26 to 32 kb (Eckerle *et al.* 2010); this genome encodes 16 nonstructural proteins (nsps), four structural proteins and several accessory proteins (Subissi *et al.* 2014a) (Fig. 1). Among the nonstructural proteins, nsp1 and nsp2 are capable of suppressing host mRNA translation and interfering with the innate immune response of host cells (Schubert *et al.* 2020; Thoms *et al.* 2020; Xu *et al.* 2022). Nsp3 and nsp4 are involved in the formation of double-membrane vesicles (DMVs) and play crucial roles in facilitating RNA transport and viral particle packaging (Huang *et al.* 2024). In addition, nsp3 is able to exert PL^{pro} activity to cleave the polyprotein pp1a (Gao *et al.* 2021) and facilitate mRNA transcript translation (Lei *et al.* 2021). Nsp5, known as the main protease (M^{pro}), cleaves the polyproteins pp1a and pp1ab into individual nonstructural proteins (Jin *et al.* 2020a). Nsp6 can form a zipper-like structure that is instrumental in enabling the transfer of information between DMVs and the endoplasmic reticulum (Ricciardi *et al.* 2022). Nsp7, nsp8, nsp9, nsp10, nsp12, nsp13, nsp14 and nsp16 can assemble into the replication and transcription complex (RTC), which is responsible for executing RNA synthesis (Wang *et al.*

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2020), unwinding (Yan *et al.* 2021a), capping (Park *et al.* 2022; Viswanathan *et al.* 2020; Yan *et al.* 2021b), backtracking and proofreading (Wang *et al.* 2025; Yan *et al.* 2021b). Nsp15 can oligomerize into hexamers, thereby exhibiting endonuclease activity (Pillon *et al.* 2021) (Table 1).

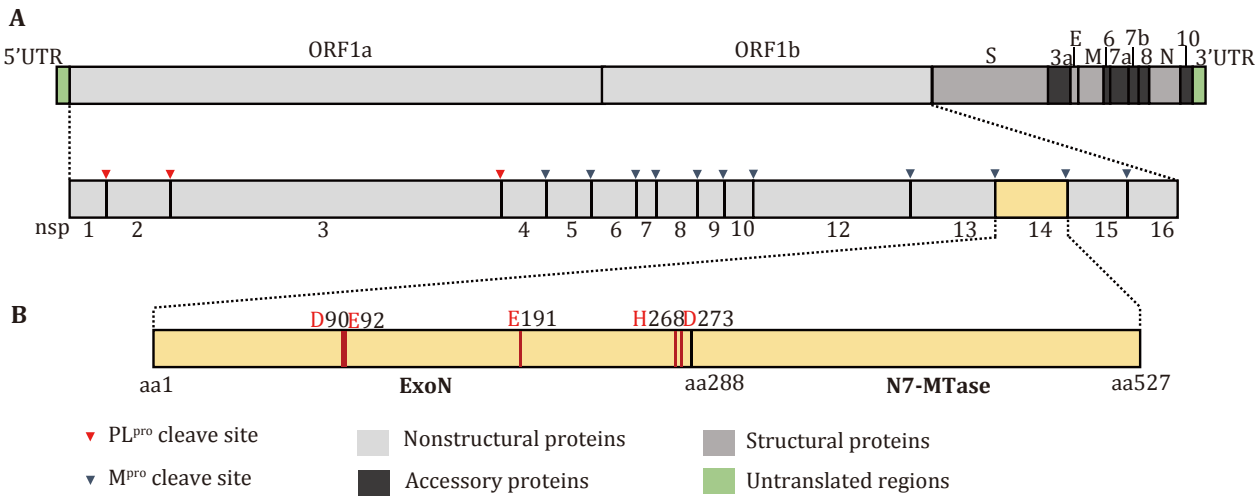

Fig. 1 SARS-CoV-2 genome organization and nsp14 ExoN residues. **A** Schematic of SARS-CoV-2 genome. ~30 kb positive single-strand RNA. Untranslated regions are shown in light green, nsp14 is shown in yellow, other nonstructural proteins are shown in light gray, structural proteins are shown in gray, accessory proteins are shown in dark gray. **B** Diagram of SARS-CoV-2 nsp14. The 1–288 amino acid (aa) in N-terminal is ExoN and the 289–527 aa in C-terminal is N7-MTase. The "DEDDH" catalytic residues in ExoN are shown as red lines

Table 1 The nonstructural proteins of SARS-CoV-2

Protein	Function	References
Nsp1	It blocks the mRNA entry channel of the 40S ribosome, inhibits twidth:10%ranslation initiation and causes degradation of host mRNAs	Bäumlin *et al.* 2025; Berlanga *et al.* 2025
Nsp2	It functions as a delivery vehicle for host proteins and disrupts intracellular host signaling	Cornillez-Ty *et al.* 2009; Zheng *et al.* 2024
Nsp3	It can exert PL^{pro} activity, promote assembly of DMV and facilitate mRNA transcript translation	Gao *et al.* 2021; Huang *et al.* 2024; Lei *et al.* 2021
Nsp4	Assembly of DMV and recruitment of RTC	Yang *et al.* 2025
Nsp5	M^{pro} activity	Jin *et al.* 2020a; Jin *et al.* 2020b
Nsp6	It forms a zipper-like structure that enables the transfer of information between DMV and ER	Ricciardi *et al.* 2022
Nsp7	Cofactor of RNA polymerase	Gao *et al.* 2020
Nsp8	Cofactor of RNA polymerase and primase activity	Gao *et al.* 2020; te Velthuis *et al.* 2012
Nsp9	It works as a substrate for RNAylation to mediate RNA capping	Park *et al.* 2022; Yan *et al.* 2022
Nsp10	It serves as a cofactor mediating both nsp16 2'O-MTase and nsp14 ExoN and N7-MTase activities	Ma *et al.* 2015; Misra *et al.* 2025
Nsp11	Important for replication	Fang *et al.* 2008
Nsp12	RNA-dependent RNA polymerase and RTase	Gao *et al.* 2020; Yan *et al.* 2022
Nsp13	Helicase and RTPase activity	Yan *et al.* 2021a
Nsp14	ExoN and N7-MTase activity	Ma *et al.* 2015
Nsp15	Endoribonuclease	Kalia *et al.* 2024
Nsp16	2'O-MTase activity	Misra *et al.* 2025

CoV nsp14 is a bifunctional enzyme. The N-terminal end contains an exoribonuclease (ExoN) domain responsible for proofreading, while the C-terminal end harbors an N7-methyltransferase (N7-MTase) domain responsible for capping (Fig. 2A). The sequence similarity between SARS-CoV-2 nsp14 and MERS-CoV nsp14 is 62.0%, whereas the similarity with SARS-CoV nsp14 reaches as high as 95.1%. The N7-MTase domain of nsp14 has an atypical methyltransferase fold featuring a conserved S-adenosylmethionine (SAM)-binding pocket and a substrate-binding site (Ma *et al.* 2015). The active site of the N7-MTase domain is situated at the interface between these two pockets, enabling the efficient transfer of a methyl group from SAM to the guanine residue of the RNA cap core (GpppA). Additionally, this domain comprises several loops and helices that contribute to its overall structural stability and substrate specificity. During the CoV life cycle, nsp14 catalyzes the methylation of the N7 position of the first guanosine monophosphate (GMP) in the cap core, resulting in the formation of the cap(0) structure (^{7Me}GpppA) (Chen *et al.* 2009).

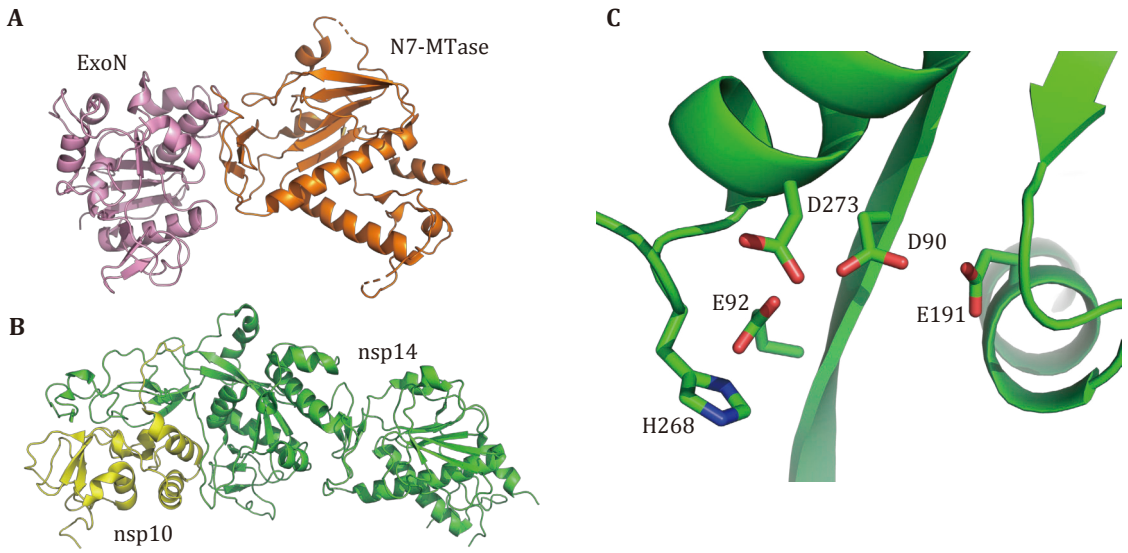

Fig. 2 Structure models of CoV nsp14 and nsp10/14 complex in cartoon diagrams. **A** Diagram of SARS-CoV-2 nsp14 structure (PDB: 7QGI). ExoN region is shown in pink, N7-MTase represents in orange. **B** Structural diagram of SARS-CoV nsp10/14 complex (PDB: 5C8U). Nsp10 is shown in yellow, nsp14 represents in green. **C** "DEDDH" catalytic residues in active pocket of SARS-CoV-2 nsp14 ExoN (PDB: 7N0B). Nitrogen atoms are shown in blue; oxygen atoms are shown in red

Unlike most RNA viruses, which have high estimated error rates, CoVs exhibit a high level of fidelity during the replication of their large genomes, which is attributable to the proofreading mechanism carried out by nsp14 ExoN (Eckerle *et al.* 2007; Minskaia *et al.* 2006). The ExoN domain of nsp14 consists of a core catalytic region that harbors the catalytic residues "DEDDH", which are essential for ExoN activity (Minskaia *et al.* 2006). Moreover, nsp14 is involved in regulating viral genome recombination (Gribble *et al.* 2021) and modulating the host's innate immune response (Becares *et al.* 2016; Rashid *et al.* 2022). Here, we comprehensively review the functional roles and structural characteristics of CoV nsp14 in the processes of cap synthesis and proofreading. In addition, we systematically delineate the potential inhibitors of nsp14 and discuss the underlying mechanisms of their effects. Our analysis highlights the importance of nsp14 in viral replication and emphasizes its potential as a target for antiviral drug development.

EXON ACTIVITY OF NSP14

As an ortholog of the *E. coli* DNA polymerase III ε-subunit, nsp14 ExoN belongs to the DEDD superfamily, which is characterized by four conserved catalytic residues, including three asparagine residues and one glutamic acid residue (Snijder *et al.* 2003). These residues cooperate with divalent metal ions to facilitate

RNA hydrolysis (Chen *et al.* 2007). During hydrolysis, nsp14 ExoN recruits two metal ions at the active site, such as Mg^{2+}, Mn^{2+} and Zn^{2+}, where one metal ion activates a water molecule for nucleophilic attack on the scissile phosphate diester and the second metal ion stabilizes the pentacoordinate phosphorane transition state through interactions with bridging and non-bridging oxygen atoms. The DEDD motif residues directly participate in metal chelation. Three aspartates coordinate to both metal ions, and the glutamate residue stabilizes the nucleophilic water molecule (Liu *et al.* 2021). This concerted action ensures efficient phosphate ester bond cleavage for RNA substrates.

In 2015, Ma *et al*. reported the first high-resolution crystal structures of the CoV nsp10/14 complex, revealing a conserved catalytic core harboring five essential residues: $_{nsp14}$D90, $_{nsp14}$E92, $_{nsp14}$E191, $_{nsp14}$H268, and $_{nsp14}$D273 (Ma *et al.* 2015) (Figs. 2B and 2C). Mutagenesis studies demonstrated that alanine substitution of these residues not only abrogated ExoN activity by >90% (Ma *et al.* 2015; Robson *et al.* 2020) but also caused defective synthesis and misdistribution of viral subgenome RNA (sgRNA) in infected cells (Minskaia *et al.* 2006). Structural analysis of the nsp10/14 complex with an RNA substrate and divalent metal ions highlighted unique features distinguishing CoV nsp14 from canonical DEDD superfamily members, including: (1) a three-stranded antiparallel β-sheet mediating nsp10-dependent allosteric activation (Imprachim *et al.* 2023); (2) another histone for stabilizing catalytic water; and (3) two additional specific zinc finger motifs (Ma *et al.* 2015). Functional characterization has further demonstrated that viruses with mutated nsp14 ExoN exhibit a higher mutation rate than WT viruses (Gribble *et al.* 2021). Additionally, mutation of nsp14 ExoN in MERS-CoV results in impaired RNA synthesis, thereby inhibiting viral proliferation (Ogando *et al.* 2020). These findings establish that the nsp14/nsp10 complex plays an essential role in maintaining RNA synthesis fidelity through its hydrolytic activity.

FACTORS INFLUENCING NSP14 EXON ACTIVITY

Nsp10

Nsp10 maintains the structural integrity and increases the enzymatic activity of nsp14 ExoN. While monomeric nsp14 exhibits basal RNA cleavage activity, nsp10 binding enhances ExoN enzymatic efficiency by a factor of 260 in SARS-CoV-2 (Riccio *et al.* 2022), primarily by stabilizing the catalytic core of ExoN. Structural analysis of the SARS-CoV-2 nsp10/14 complex has revealed that nsp10 interacts with the N-terminal domain of nsp14 via a conserved subdomain composed of four helices and two β-strands, forming a handshake-like interface (Lin *et al.* 2021). These interactions stabilize the ExoN active site by coordinating the DEDD residues ($_{nsp14}$D90, $_{nsp14}$E92, $_{nsp14}$E191, $_{nsp14}$D273) into a functional configuration and remodeling the RNA-binding channel to enhance substrate binding. In the absence of nsp10, the catalytic pocket of nsp14 undergoes partial conformational collapse, impairing metal ion-mediated hydrolysis (Ferron *et al.* 2018). Mutagenesis of interface residues (*e.g.*, $_{nsp14}$D8A and $_{nsp14}$K7A) results in a >90% reduction in viral titers (Grimes *et al.* 2025), confirming the essential role of this protein–protein interaction in viral replication. These findings highlight nsp10 as a critical cofactor that induces allosteric activation of nsp14 ExoN through structural stabilization and functional enhancement.

Nsp14 zinc finger

Compared with other members of the DEDD superfamily, nsp14 contains two additional specific zinc finger (ZnF) motifs in ExoN (Chen *et al.* 2007; Robson *et al.* 2020). Zinc fingers are small, independently folded domains and contain conserved cysteine and histidine residues, which coordinate with Zn^{2+} and assist in the recognition of RNA or DNA substrates. In SARS-CoV nsp14 ExoN, ZnF1 (C210 to H229) mediates stable complex formation with nsp10, a prerequisite for ExoN activation, and ZnF2 (C261 to H264) directly supports catalytic function through RNA substrate positioning (Ma *et al.* 2015). Functional studies have demonstrated that mutation of H229C in ZnF1 aborts the replication of MERS-CoV, which might be attributable to the disruption of interactions between nsp10 and nsp14 and further inhibition of ExoN activity (Ogando *et al.* 2020; Robson *et al.* 2020). In addition, a mutation of H229C in ZnF1 leads to an inhibition of antiviral responses to α-CoV in host cells, thereby inducing a 10-fold increase in the virus titer compared with that of the wild type (Becares *et al.* 2016). Additionally, ZnF1 mutations trigger nsp14 protein insoluble in prokaryotic expression systems (Ma *et al.* 2015), suggesting a critical function of zinc finger 1 in maintaining the stability of nsp14. In contrast, ZnF2 mutants impair proofreading activity entirely, leading to an abolishment of MERS-CoV viability (Ogando *et al.* 2020). Future studies should investigate how ZnFs serve as antiviral targets to inhibit nsp14 ExoN activity.

Metal ions

As reported in a previous study (Bouvet *et al.* 2012), the ExoN activity of CoV nsp14 is strictly dependent on divalent metal ions. Biochemical studies have demonstrated that Mg^{2+} is the preferred cofactor for optimal catalytic efficiency, although Zn^{2+} and Mn^{2+} can partially substitute for Mg^{2+} in promoting RNA hydrolysis (Bouvet *et al.* 2012). However, replacement of Mg^{2+} with Ca^{2+} almost entirely inhibits the hydrolytic activity of ExoN, resulting in no cleavage of RNA substrates (Lin *et al.* 2021). With respect to the underlying reason, there is not yet a well-defined molecular basis. The cryo-EM structures of the Ca^{2+}-bound SARS-CoV-2 nsp10/14 complex and the Mg^{2+}-bound SARS-CoV-2 nsp10/14 ($_{nsp14}$E191A) mutation reveal conserved coordination in the catalytic center, with only minor conformational differences in the RNA substrate and protein residue side chains (Liu *et al.* 2021). This structural conservation is similar to observations in other nuclease families (Lee *et al.* 2005; Rychlik *et al.* 2010), suggesting that the inhibitory effect of Ca^{2+} substitution cannot be fully rationalized by static conformational changes. Instead, several chemical factors should be considered, including: (1) the physicochemical properties of the metal ions and (2) the interactions of the metal ions with the RNA substrate as well as with the catalytic water molecules. Generally, Mg^{2+} typically forms hexa-coordinated complexes (Babu *et al.* 2013; Wolf and Cittadini 2003), whereas the coordination number of calcium is 6–10 (Maguire and Cowan 2002). Therefore, calcium has a high ligand exchange constant and is transferred more easily between two ligands, which may stabilize the intermediate state of RNA hydrolysis and disrupt efficient catalysis. Furthermore, whereas Mg^{2+} coordinates with two carboxylate groups in the active site, Ca^{2+} forms an additional bond with the carboxylate of another asparagine, thereby tightening its binding to the enzyme and hindering the hydrolysis of the RNA substrate (Moomaw and Maguire 2008). Notably, the larger atomic radius of Ca^{2+} also affects hydration dynamics. Whereas Mg^{2+} has a hydrated radius approximately 400 times larger than its dehydrated radius (Babu *et al.* 2013; Moomaw and Maguire 2008), the hydrated radius of Ca^{2+} is only 25 times greater than the dehydrated radius (Moomaw and Maguire 2008). This reduces the possibility of recruiting catalytic water molecules to the active site, which is critical for nucleophilic attack on the scissile phosphate bond.

Molecular dynamics simulations by Rosta *et al.* further demonstrated that Ca^{2+} substitution increases the reaction barrier by altering the geometry of the active site, thereby abolishing the catalytic activity of ExoN (Rosta *et al.* 2014). Additionally, Ca^{2+} ions increase the distance between the catalytic water and the scissile phosphate and disrupt the optimal positioning of the catalytic water relative to the scissile phosphate, which increases the energetic cost of hydrolysis (Rosta *et al.* 2014). Notably, the inhibitory effect of Ca^{2+} is not universally conserved across nuclease families. For example, *E. coli* RNase I displays Ca^{2+}-dependent activity on double-stranded RNA (dsRNA), which cannot be recapitulated by other divalent ions (Grünberg *et al.* 2021). These findings suggest that metal dependence is shaped by evolutionary adaptations to distinct biological contexts. Further investigations into the dynamic interplay among metal ions, protein residues, and RNA substrates will be critical for understanding the underlying mechanism.

SUBSTRATE PREFERENCE OF NSP14 EXON

Biochemical assays by Minskaia *et al.* revealed that SARS-CoV nsp14 ExoN displayed sequence-independent hydrolytic capability toward both single-stranded RNA (ssRNA) and dsRNA substrates (Minskaia *et al.* 2006) while exhibiting negligible activity toward DNA. Subsequent studies on SARS-CoV-2 nsp14 corroborated these findings (Baddock *et al.* 2022), demonstrating comparable hydrolytic efficiency for ssRNA and dsRNA. However, this result was challenged by Tyler *et al.*, who employed single-turnover kinetic analysis to reveal a 125-fold faster hydrolysis rate for dsRNA than for ssRNA. This discrepancy highlights the critical role of assay conditions in determining observed substrate specificity. Moreover, Tyler *et al.* demonstrated that dsRNA containing a 3'-terminal mismatch was hydrolyzed less efficiently than perfectly base-paired dsRNA (Dangerfield and Johnson 2022). In contrast, Mickaël *et al*. reported that an RNA substrate with a 3'-end mismatch competes with the base-paired substrate more efficiently (Bouvet *et al.* 2012), indicating that a mismatched 3'-end base pair might be the preferred substrate rather than a perfectly matched substrate, which is consistent with the findings of Liu *et al*. (Liu *et al.* 2021). Cryo-EM structures of the SARS-CoV-2 nsp10/14 complex bound to mismatched dsRNA revealed that the catalytic center accommodates mismatched base pairs through ion-mediated interaction networks (Liu *et al.* 2021). Specifically, the side chains of DEDD residues form bridging interactions with divalent metal ions, the mismatched base, and the scissile phosphate and maintain substrate positioning. This binding mode explains the ability of CoV nsp14

ExoN to process mismatched substrates. Additionally, the preference of the mismatched pair by nsp14 ExoN might be attributed to the greater energy required to break this base pair, which might lead to the preference of CoV ExoN for dsRNA substrates with a 3'-end mismatch instead of a perfectly matched substrate.

Moreover, SARS-CoV-2 nsp14 ExoN exhibited negligible preference for different mismatched base pairs. Single mismatches, including A:G, A:A, and A:C pairs, are efficiently excised by nsp14 ExoN (Bouvet *et al.* 2012). Importantly, the number of 3'-terminal mismatches rarely impacts the hydrolytic efficiency of nsp14 ExoN (Dangerfield and Johnson 2022). These observations suggest that ExoN activity is independent of the nucleotide misincorporated at the 3'-end. Future investigations should focus on determining how this property influences viral replication fidelity and antiviral drug development strategies.

INTERACTIONS BETWEEN NSP14 AND OTHER NONSTRUCTURAL PROTEINS

To date, most studies have focused on exploring the nsp10/14 complex but have neglected that ExoN presumably functions within the RTC containing helicases, RdRp, and potentially other viral nonstructural proteins, which might affect the kinetics of RNA hydrolysis. Pull-down assays by Subissi *et al*. first revealed a direct interaction between nsp12 and nsp14 in SARS-CoV, which did not alter the enzymatic activities of RdRp of nsp12 or nsp14 MTase or ExoN (Subissi *et al.* 2014b). Further studies revealed that nsp10 increases ExoN activity when incorporated into the nsp7/8/12/14 complex, indicating that nsp10 might not only function as a scaffold within the nsp7/8/10/12/14 supercomplex but also serve as a dissociable activator of monomeric nsp14 (Subissi *et al.* 2014b).

Structural studies provided mechanistic insights into these interactions. A flexible hinge observed in CoV nsp14 separates the ExoN and N7-MTase domains (Ferron *et al.* 2018), which could act as a molecular switch, enabling dynamic interactions with diverse protein partners and RNA substrates. Biochemical assays by Liu *et al*. revealed that nsp8 increased the ExoN activity of SARS-CoV-2 nsp14, and copurification through size-exclusion chromatography (SEC) resulted in the formation of the nsp8/10/14:RNA complex (Liu *et al.* 2021). However, cryo-EM analysis of the nsp10/14-RNA complex revealed no nsp8 density, suggesting that transient interactions stabilize substrate binding without the formation of a stable complex. In addition, cryo-EM analysis of SARS-CoV-2 Cap(0)-RTC by Yan *et al.* revealed that nsp9 and the NiRAN domain of nsp12 recruit nsp10/14 to form a functional complex responsible for yielding a cap(0) structure (^{7Me}GpppA) at the 5' end of mRNA (Yan *et al.* 2021b) (Fig. 3A). Moreover, the dimeric form of Cap(0)-RTC may serve as a model in which the nsp14 exon executes proofreading of the mismatched RNA in coordination with nsp12 through a "backtracking" mechanism (Fig. 3B). In this model, when the RTC senses the misincorporated nucleotide in the nascent RNA, the misincorporated nucleotide can be translocated upstream by the nsp13 helicase, allowing nsp14 ExoN to excise the mismatched nucleotide at its catalytic center (Fig. 3C).

NSP14 EXON-MEDIATED PROOFREADING

On the basis of ExoN activity, nsp14 ExoN is supposed to conduct proofreading during CoV RNA synthesis, thereby preventing the polymerase from crossing an "error threshold" above which it would render viral replication inviable. The proofreading activity of nsp14 is critical for maintaining genome stability, as RNA viruses lacking ExoN (*e.g*., HIV) exhibit a mutation rate of 10^{-4}–10^{-3} (Nowak 1990), whereas CoVs with intact ExoN activity have a significantly lower mutation rate of 10^{-6}–10^{-5} (Blank *et al.* 1986; de Mercoyrol *et al.* 1992; Rosenberger and Hilton 1983). Indeed, CoVs are the only RNA viruses known to possess ExoN. Lance *et al.* reported that CoV mutants harboring inactive nsp14 ExoN display obvious growth defects, including a 21-fold increase in mutation frequency during genome replication of SARS-CoV (Eckerle *et al.* 2010) and a 31.6- to 100-fold reduction in the plaque phenotype and titers of SARS-CoV-2 compared with those of the parental virus (Ogando *et al.* 2020). Substitutions of SARS-CoV nsp14, such as R310A and F426A, in nsp14 abolished viral proliferation, whereas the H424A mutation resulted in a loss-of-function phenotype (Ogando *et al.* 2021). Evolutionary analyses of CoV nsp14 sequences further highlight the adaptive importance of ExoN. Hassan *et al*. identified 962 mutations across global isolates of SARS-CoV-2, with 548 (56.7%) classified as neutral mutations and 414 (43.3%) considered deleterious (Hassan *et al.* 2024). These deleterious mutations cluster in conserved residues critical for RNA binding and catalysis, impairing the function or production of the nsp14 protein. Conversely, neutral mutations can maintain the normal activities of nsp14. Notably, a balance between deleterious and neutral mutations in nsp14 is critical

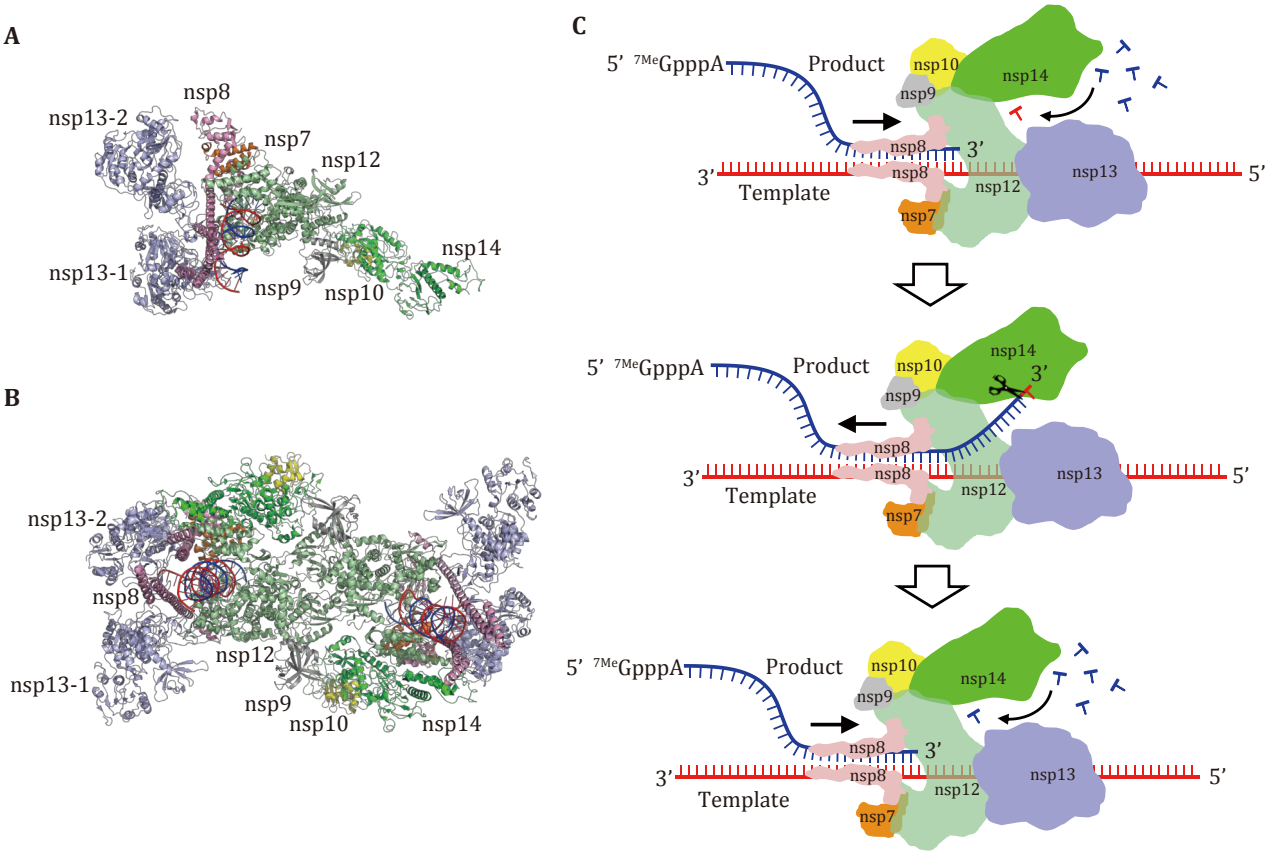

Fig. 3 The replication and proofreading complex of CoV. **A** Structure model of monomeric cap(0)-RTC of SARS-CoV-2 (PDB: 7EIZ). **B** Structure models of dimeric cap(0)-RTC (PDB: 7EGQ) of SARS-CoV-2. **C** Model of proofreading complex of CoV. Nsp7/8 promote nsp12-mediated replication and transcription of the genome and sgmRNA. Nsp13 is responsible for unwinding the dsRNA. Nsp9 acts as a mediator for nsp12-catalyzed cap formation. Nsp14 ExoN proofreads the nascent RNA strand and excises misincorporated nucleotides with its co-factor nsp10. Nsp7, nsp8, nsp12, nsp13 are shown in orange, pink, palegreen and lightblue, separately. Nsp9, nsp10 and nsp14 are represent in gray, yellow and green, respectively. Template RNA is shown in red and product RNA is shown in blue. Arrow presents the movement direction of the proofreading complex

for viral fitness and adaptation. While ExoN activity can restrict beneficial mutations, complete loss of proofreading leads to error catastrophe. This delicate equilibrium might contribute to coronavirus persistence in host populations. Future studies should explore how ExoN modulates this balance, which may interact with other replication factors that are critical for antiviral drug development.

PROOFREADING MECHANISM IN EUKARYOTIC CELLS

Compared to viral RNA polymerase, RNA polymerase in eukaryotic cells generally exhibits higher fidelity. The estimated error rate of eukaryotic RNA polymerase (RNAP) is typically less than 10^{-5} (Blank *et al.* 1986; de Mercoyrol *et al.* 1992). Research has shown that RNAP modulates the conformation of its active center to preferentially bind the correct nucleoside triphosphate (NTP) substrate (Opalka *et al.* 2003; Thomas *et al.* 1998). Upon entry of the correct NTP, it binds to the open active center and forms a Watson-Crick base pair with the DNA template (Vassylyev *et al.* 2007; Westover *et al.* 2004). Subsequently, the initiation loop (TL) of RNAP undergoes a structural transition from an inactive open state to an active closed state (Kireeva *et al.* 2008; Vassylyev *et al.* 2007; Wang *et al.* 2006). In contrast, mismatched NTPs, which are unable to form proper base pairs or establish essential interactions with key residues in the TL domain, fail to induce efficient folding of TL. This results in a reduction of TL affinity for mismatched NTPs by more than 100 folds (Yuzenkova *et al.* 2010). This mechanism enables RNAP to effectively distinguish between correctly paired and mismatched NTPs, thereby ensuring transcriptional

accuracy.

In eukaryotic cells, when a mismatched NTP is incorporated into the RNA terminus by RNA polymerase II (Pol II), it remains in the active site pocket. This position overlaps with the triphosphate-binding site of the incoming NTP, thereby preventing the subsequent NTP from binding to the active center and halting the RNA elongation process (Sydow *et al.* 2009). The cessation of elongation prolongs the residence time of Pol II in the pre-translocation state, increasing the likelihood of RNA 3' end shifting and thermodynamically favoring the occurrence of transcript reversion.

Eukaryotic RNAP can not only detect the incorporation of mismatched NTPs but also possesses exonuclease activity. Typically, when base-paired NTP enter the reaction center of RNAP, it is rapidly incorporated into the RNA chain. Conversely, mismatched NTPs are recognized and excised by the exonuclease domain of RNAP (Kettenberger *et al.* 2003, 2004; Kuhn *et al.* 2007). In mammalian transcription elongation complexes, the removal of mismatched nucleotides is carried out by Pol II, with its exonuclease activity being enhanced by the transcription elongation factor TFIIS (Kettenberger *et al.* 2003, 2004). For nucleotide hydrolysis, the phosphodiester bond should align with the catalytic site of the exonuclease domain, which is facilitated by RNAP backtracking. Studies have demonstrated that the RNA undergoes backtracking after the excision of a mismatched nucleotide, creating an empty NTP binding site and allowing RNAP to resume active transcription. Notably, Pol II preferentially removes mismatched nucleotides at the RNA terminus with the assistance of TFIIS. However, Pol II relies on its subunit Rpb9 rather than TFIIS itself *in vivo*, with TFIIS primarily facilitating the recruitment of catalytic metal ions or water molecules necessary for hydrolysis (Damsma *et al.* 2007; Kireeva *et al.* 2008; Wang *et al.* 2009).

However, RNA polymerases typically lack such a backtracking and proofreading mechanism during viral transcription. For instance, SARS-CoV-2 RNA-dependent RNA polymerase (RdRp) exhibits similar incorporation efficiency of mismatched nucleotides compared to that of base paired ones (Petushkov *et al.* 2023). Although the low fidelity contributes to genomic diversity, enabling the virus to adapt to various environmental and selective pressures, it also increases the risk of lethal mutations. Therefore, several nucleotide analogues have been developed as antiviral agents, such as remdesivir (Ferner and Aronson 2020) and azvudine (Wang *et al.* 2014). Research has indicated that RdRp exhibits a higher propensity to bind the triphosphate form of remdesivir compared to ATP, incorporating it into nascent RNA chains and thereby inhibiting further elongation and viral genome transcription (Dangerfield *et al.* 2020). However, some viruses have evolved proofreading mechanisms to preserve genetic stability by removing mismatched nucleotides via exonuclease activity. To date, CoVs are the only known RNA viruses to possess such a backtracking and proofreading mechanism.

EFFECTS OF NSP14 EXON ON NAS

It has been confirmed that CoVs with inactive nsp14 ExoN are more prone to accumulate mutations during genome replication. Compared with the WT virus, engineered SARS-CoV harboring the nsp14-ExoN mutation presented a 21-fold increase in mutation frequency during *in vitro* replication in cell culture (Eckerle *et al.* 2010). Notably, the ExoN mutation of CoV increases its sensitivity to antiviral nucleotide analogs (NAs) compared with that of the WT virus, such as a 4.5-fold increase in response to remdesivir (Shannon *et al.* 2020) and a 100-fold increase in response to ribavirin (Smith *et al.* 2013), indicating an improvement in drug efficacy when ExoN is abolished. Regrettably, to date, no drugs specifically targeting nsp14 ExoN have become available for clinical use. To identify potential ExoN inhibitors, Liang *et al*. screened over 5000 compounds, including FDA-approved drugs, natural products, and a library of antiviral compounds, on the basis of the crystal structure of the SARS-CoV-2 nsp10/14 complex (Liang *et al.* 2024). A variety of potential inhibitors that target nsp14 ExoN, such as conivaptan and hesperidin, have been identified. These compounds can significantly inhibit viral viability either under single agent treatment or in combination with RdRp inhibitors (Hoever *et al.* 2005; Khater *et al.* 2021). Although ExoN inhibitors alone may sufficiently impact viral genome replication, their combination with RdRp inhibitors can more effectively restrict viral proliferation. Given that ExoN is highly conserved among CoVs and has no homologous counterpart in the host genome, nsp14 ExoN represents an important target for the development of novel antiviral drugs.

As the cornerstone of antiviral therapy, more than 30 NAs have been approved by the FDA for clinical use over the past 50 years (Roy and Agrofoglio 2022). Generally, NAs work in a 5'-triphosphorylated form and impede viral replication by targeting viral DNA/RNA polymerase or reverse transcriptase. During the COVID-19 pandemic, a re-evaluation of existing antiviral drugs revealed that several NAs, such as remdesivir (Spinner *et al.* 2020) and molnupiravir (Harris *et al.* 2025),

exhibit potent antiviral effects on SARS-CoV-2. Mechanistically, NAs can be recognized by CoV RdRp and incorporated into the RNA product strand, thereby inducing termination of RNA elongation (Moeller *et al.* 2022) or mutation (Kabinger *et al.* 2021) in nascent RNA. However, NAs incorporated at the 3'-end of RNA can be recognized by the proofreading mechanism of cap(0)-RTC and excised by nsp14 ExoN, thus reducing the efficacy of NAs. For example, remdesivir, an NA approved by the FDA for clinical use in the treatment of COVID-19 (Maxmen 2020), can be removed by nsp14 ExoN when it is incorporated into the 3'-end of RNA (Chinthapatla *et al.* 2023; Wang *et al.* 2022b), which is also supported by Liu *et al*. (Liu *et al.* 2021). Notably, in MHVs with nsp14 ExoN knockout, the susceptibility of the virus to remdesivir was significantly greater than that of the WT virus (Agostini *et al.* 2018), suggesting that product RNA containing RMP could be a substrate for CoV ExoN. Conversely, a previous study by Zhang *et al*. demonstrated that when the product RNA with 3'-end remdesivir is located in the active pocket of nsp14 ExoN, remdesivir might prolong the distance between the hydroxyl oxygen at the 3' position of the second nucleotide in the 3' end and Mg^{2+}. This results in obvious base bending in remdesivir compared with adenine nucleotides, which hinders the interaction between remdesivir and ExoN and helps remdesivir escape nsp14 ExoN cleavage (Zhang *et al.* 2021). Unlike other chain terminators, remdesivir has a specific termination site at position i+3 (Gordon *et al.* 2020), which leads to a distortion of the position of the RNA and hampers the translocation of the next nucleotide (Wang *et al.* 2020). Nevertheless, whether remdesivir at position i+3 can be sensed by the RTC backtracking model to trigger nsp14 proofreading remains unclear. Notably, molnupiravir, another effective NA for CoV (Barnard *et al.* 2004; Pyrc *et al.* 2006), can prevent nsp14 ExoN. Unlike remdesivir, molnupiravir increases the mutation frequency of the viral genome rather than directly terminating RNA synthesis (Kabinger *et al.* 2021), suggesting that molnupiravir may evade proofreading for direct incorporation into nascent RNA without inhibiting RNA synthesis. Molnupiravir has been shown to inhibit ExoN-mutant CoV and the wild-type virus equally (Agostini *et al.* 2018). Additionally, molnupiravir remarkably inhibits coronaviruses that are resistant to remdesivir (Sheahan *et al.* 2020), indicating that molnupiravir may have the ability to escape from nsp14 ExoN. Notably, molnupiravir has mutagenic effects on host genes when incorporated into RNA products (Chebotareva and Ditiatev 1988; Zhou *et al.* 2021), suggesting a lack of selectivity for molnupiravir between viral and host polymerases. Therefore, understanding the mechanism by which nsp14 ExoN recognizes nucleotide analogs and rationally designing nucleotide analogs to prevent proofreading are highly warranted for the development of anti-CoV nucleotide analogs.

MTASE ACTIVITY OF NSP14

The C-terminus of nsp14 is the N7-MTase domain and contains a D×G SAM motif that is conserved in CoV and capable of forming a cap(0) structure (^{7Me}GpppA). In eukaryotic cells, both host and viral RNA lacking 5'-cap structures undergo severe degradation with the assistance of several ExoN. The cap structure at the 5' end of RNA can block the hydrolysis induced by the host 5'-3' ExoN, which plays an important role in maintaining RNA stability. Importantly, the capped RNA is not recognized by the host cell as "foreign RNA" and can therefore escape the host cell immune response (Pan *et al.* 2022). Compared with the RNA virus MTase, which is characterized by a canonical "Rossmann fold", the N7-MTase of CoV nsp14 typically features an uncanonical "Rossmann fold" (Ma *et al.* 2015). Indeed, the Rossmann fold consists of six β-strands intercalated by four α-helices, of which the last strand may undergo modification and extend into the seventh β-strands (Chouhan *et al.* 2019; Schubert *et al.* 2003). However, the N7-MTase of CoV nsp14 features a central five-stranded predominantly parallel β-sheet rather than seven β-strands (Imprachim *et al.* 2023) and therefore does not belong to any class of SAM-dependent MTases. The crystal structure of the SARS-CoV nsp10/14 complex revealed two motifs critical for RNA capping: (1) a typical SAM-binding motif (DxGxPxG/A), of which SAM is the methyl donor of nsp14 N7-MTase for yielding the ^{7Me}GpppA structure (Ogando *et al.* 2021); (2) nsp14 residues Y420 to T428 that form a constricted pocket holding the cap's GTP moiety (Ma *et al.* 2015) (Fig. 4). In MHV and SARS-CoV, mutations in the DxG motif abolish N7-MTase activity, which clearly has a detrimental effect on replication without disrupting ExoN activity (Case *et al.* 2016). Although the ExoN domain is not directly involved in SAM binding by N7-MTase (Chen *et al.* 2009), the interactions between these two domains are critical for maintaining MTase activity. Interference by residues at the interface, such as Q262, W86 and R84, may affect the structure of nsp14 and lead to a decrease in MTase activity. For example, a previous study revealed that the R84A and W86A mutations in nsp14 ExoN obviously attenuated N7-MTase activity, and the activity was even abolished after truncations of the N-terminus between amino

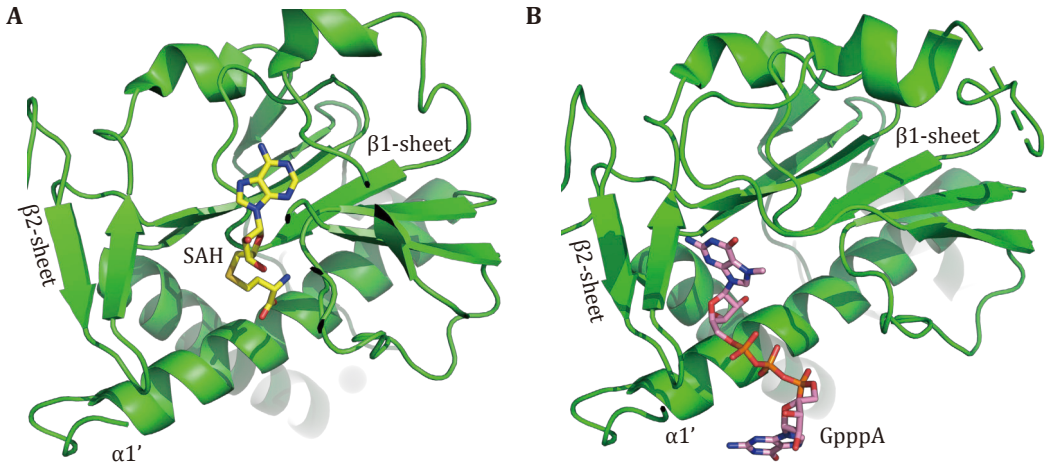

Fig. 4 Ligand-binding sites in the nsp14 N7-MTase domain of SARS-CoV-2. Substrates SAH (**A**) (PDB: 7R2V) and GpppA (**B**) (PDB: 7QIF) bind in pockets formed by the β1-sheet, β2-sheet, and α1'. Nsp14 N7-MTase domain is shown in green, SAH and GpppA represent in yellow and pink, separately

acids 78 and 90 *in vitro* (Chen *et al.* 2009, 2013). These results may also indicate that the two enzymatic domains of nsp14 are structurally interconnected, with N7-MTase activity depending on the integrity of the N-terminal ExoN domain.

Cap formation is a tightly regulated and coordinated process consisting of four consecutive enzymatic reactions. After the GpppA structure is yielded by RNAylation and deRNAylation, nsp14 or nsp16 is recruited into the RTC, where it methylates GpppA to produce ^{7Me}GpppA and ^{7Me}GpppA$_{2'OMe}$ in sequence (Park *et al.* 2022). In a previous study, Mickaël *et al.* demonstrated that the nsp10 surface involved in the nsp10–nsp14 interaction overlaps with the nsp10–nsp16 interaction surface (Bouvet *et al.* 2014). Nsp10 molecules may thus act as a platform that recruits nsp14 or nsp16 to the replication-transcription complex to either increase nsp14 ExoN activity or switch on nsp16 2'-O-MTase activity. Thus, nsp10/14 and nsp10/16 complexes may coexist to function separately. However, a recent study by Alex *et al*. revealed that nsp14, nsp10, and nsp16 can form a heterotrimer complex upon significant allosteric changes (Matsuda *et al.* 2024). Although a high-resolution structure of the nsp10/14/16 complex was not obtained, the low-resolution structural data confirmed the existence of the heterotrimer in solution together with the biochemical data. Further study to determine the high-resolution structure of the nsp10/14/16 complex is important for understanding the RNA capping of CoV and is helpful for developing novel therapeutic approaches for CoV (Lin *et al.* 2022).

POTENTIAL INHIBITORS OF NSP14

Nsp14 is considered a potential drug target because of its dual enzymatic activities as both an MTase and ExoN (Rona *et al.* 2022; Tahir 2021). Mutations in either domain severely interrupt viral replication, highlighting their essential roles in the CoV life cycle. ExoN-mediated proofreading is important for viral viability, as it cleaves mismatched nucleotides during RNA synthesis and confers resistance against nucleotide analogs, thereby impairing the efficacy of those nucleotide analogs. Moreover, the MTase activity of nsp14 enhances viral fitness by capping nascent RNA to protect against degradation and to facilitate immune evasion. Notably, both the ExoN and MTase domains lack orthologs in the human genome, minimizing the risk of off-target effects of nsp14 inhibitors and positioning them as attractive candidates for the development of small-molecule inhibitors.

Given the ExoN activity of nsp14, inhibitors can suppress the cleavage of the RNA substrate through three mechanisms: (1) chelating metal ions from the active site of ExoN to inhibit recruitment of the catalytic water molecule; (2) occupying the catalytic pocket of nsp14 ExoN; and (3) interacting at the nsp10/14 interface to hinder nsp10 binding (Table 2). Several studies have reported small-molecule inhibitors that target nsp14 ExoN, such as aurintricarboxylic acid (ATA) (Canal *et al.* 2021), inhibitor 1c (Asthana *et al.* 2023) and raltegravir (Baddock *et al.* 2022). For example, ATA potently inhibits the ExoN activity of the SARS-CoV-2 nsp10/14 complex (Canal *et al.* 2021). As a nonspecific metal chelator (Hernández *et al.* 2022; Liu *et al.* 2014), ATA might disrupt metal ion coordination

and RNA substrate recognition by chelating structurally and functionally critical metal ions. In addition, Abhishek *et al.* synthesized a novel nitrocatechol compound, inhibitor 1c, that exhibits antiviral activity against SARS-CoV-2 by inhibiting nsp14 ExoN (Asthana *et al.* 2023). Molecular docking simulations revealed that inhibitor 1c interacts with the catalytic residues that are critical for substrate interaction, interfering with RNA substrate binding to the ExoN catalytic pocket and thereby blocking RNA hydrolysis (Asthana *et al.* 2023). Given that nsp10 clearly increases the ExoN activity of nsp14 in CoVs, Ravindra *et al*. recently identified two compounds (compounds 4 and 7) that target nsp10 to disrupt the nsp10/14 protein–protein interaction (PPI), thereby inhibiting ExoN activity and exerting antiviral effects against SARS-CoV-2 (Jumde *et al.* 2025). However, these compounds exhibit higher IC_{50} values than other nsp14 ExoN inhibitors, such as ATA (compound 4: 1505 μmol/L, compound 7: 1148 μmol/L, ATA: 10.3 μmol/L) (Canal *et al.* 2021; Jumde *et al.* 2025). Therefore, further optimization of these compounds is necessary to improve their activity and ADMET properties. Additionally, several NAs, such as tenofovir and daclatasvir, exhibit resistance to nsp14 ExoN (Wang *et al.* 2022b). These compounds can not only be incorporated into nascent RNA to terminate the RNA polymerase reaction (Chien *et al.* 2020; Sacramento *et al.* 2021) but also prevent the cleavage of these nucleotide analogs by attenuating ExoN activity (Wang *et al.* 2022a, 2022b). Although these NAs sufficiently inhibit SARS-CoV-2 (DeJong *et al.* 2021; Kow *et al.* 2022; Şanlıdağ İşbilen *et al.* 2024), evidence is limited to support their clinical use, and several potential risks might exist for these untested drugs. For example, as an effective approach in the treatment of HIV and hepatitis B virus (Park *et al.* 2020), tenofovir might cause HIV resistance if it is utilized as an experimental therapy for COVID-19 (DeJong *et al.* 2021). Further studies should consider these untested drugs and other generic antiviral therapies in animal models and clinical therapeutic trials.

Table 2 Inhibitors showing promise against CoVs

Name	Target	Function	Reference
Aurintricarboxylic acid	ExoN	Chelating metal ions from the active site of exon	Canal *et al.* 2021
Inhibitor 1c	ExoN	Occupying the catalytic pocket of exon	Asthana *et al.* 2023
Raltegravir	ExoN	Occupying the catalytic pocket of exon	Baddock *et al.* 2022
Compound 4	ExoN	Interacting at the nsp10/14 interface	Jumde *et al.* 2025
Tenofovir	ExoN	Serving as NA that can evade exon cleavage	Wang *et al.* 2022b
Daclatasvir	ExoN	Serving as NA that can evade exon cleavage	Wang *et al.* 2022b
Ds0464	MTase	SAM analogs to inhibit RNA capping	Devkota *et al.* 2021
Mti013	MTase	Adenosine analogues	Chen *et al.* 2024
Compound 7	MTase	Adenosine analogues	Ahmed-Belkacem *et al.* 2024
Compound 25	MTase	Sulfonamide-based bisubstrate analogues	Ahmed-Belkacem *et al.* 2022
Trifluperidol	MTase	Gpppa cap analogue	Basu *et al.* 2021
Tdi-015051	MTase	Uncharged, noncovalent SAM analogs	Meyer *et al.* 2025

Efforts have also focused on developing inhibitors of nsp14 MTase, as its role in cap formation of viral RNA is critical for evading the host innate immune response and escaping from RNA hydrolysis by 5'-3' ExoN in host cells, highlighting its importance and potential status as an attractive drug target (Singh *et al.* 2023). Thus far, most reported small-molecule inhibitors of nsp14 MTase are SAM analogs. While these compounds potently inhibit nsp14 MTase activity (Ahmed-Belkacem *et al.* 2020, 2022; Devkota *et al.* 2021), their hydrophilicity hinders their ability to cross cell membranes (Kottur *et al.* 2023). Moreover, SAM analogs often lack selectivity between coronavirus and human MTases, limiting their use in clinical therapy. In addition to SAM analogs, adenosine analogs and sulfonamide-based bisubstrate analogs (Hausdorff *et al.* 2023), such as MTI013 (Chen *et al.* 2024), compound 7 (Ahmed-Belkacem *et al.* 2024), compound 25 (Ahmed-Belkacem *et al.* 2022) and trifluperidol (Basu *et al.* 2021), have been identified as nsp14 MTase inhibitors useful for treating CoV infection. These compounds occupy the SAM-binding cavity in nsp14 MTase and block SAM binding as well as

cap formation. As an adenosine analog, MTI013 selectively inhibits nsp14 with little or no activity against other SAM-dependent methyltransferases (Chen *et al.* 2024). Further studies are necessary to focus on structural modifications, druggability studies, and investigations into the mechanism of action. In a recent study, Cindy *et al.* developed TDI-015051 as a potent inhibitor of nsp14 MTase, which suppressed the viability of SARS-CoV-2 *in vitro* and *in vivo* (Meyer *et al.* 2025). As an uncharged, noncovalent inhibitor, TDI-015051 overcomes the membrane permeability limitations of SAM analogs. This novel class of inhibitors, which disrupts the binding of MTase-specific substrates, may complement existing SARS-CoV-2 antivirals and offer new pathways for antiviral drug development.

CONCLUSIONS AND PERSPECTIVES

Over the past three decades, multiple epidemic outbreaks caused by distinct CoVs have emerged, including SARS in 2002, MERS in 2012, and SARS-CoV-2 in 2019. Given their capacity for mutation, recombination, and cross-species infection, CoVs are highly likely to re-emerge in the future. As a bifunctional enzyme, nsp14 plays critical roles in the cap formation and proofreading of viral RNA. As reported in this review, nsp14 can be recruited by the RTC to form a supercomplex, facilitating catalysis from the cap core (GpppA) to the cap(0) structure (^{7Me}GpppA). However, the dynamic process underlying cap(0) structure formation remains undefined. Although studies have yielded a model for the interaction of GpppA and SAM at the nsp14 MTase active site, a mechanistic description of how nsp14 MTase catalyzes native RNA with a cap core is lacking. In addition, the proofreading mechanism involving nsp14 ExoN is not fully understood. Generally, nsp14 ExoN rarely shows a preference for a specific nucleotide substrate, yet certain NAs can evade ExoN cleavage, potentially due to the inability of the RTC to sense their incorporation into nascent RNA and undergo backtracking. Backtracking is a prerequisite for the occurrence of proofreading, necessitating functional and structural investigations into how the RTC senses abnormal RNA incorporation and assists nsp14 in excising mismatched nucleotides. Clarifying these mechanisms will be essential for developing targeted antiviral strategies against current and future CoV threats.

As nsp14 is one of the most evolutionarily conserved CoV proteins, its structural and functional properties represent promising targets for the development of inhibitors. While several small-molecule inhibitors targeting nsp14 MTase and ExoN have demonstrated potent activity *in vitro*, their efficacy and potential risks remain poorly characterized *in vivo*, necessitating further preclinical and clinical research. Notably, NAs that can both terminate RNA polymerase reactions and resist ExoN-mediated excision provide a feasible strategy for antiviral design. However, the recognition mechanism of nsp14 ExoN for NAs remains unclear, impeding rational optimization of these compounds. Existing studies have described ExoN-mediated hydrolysis of NAs at the functional level but have not provided structural insights into how NAs interact with the catalytic pocket or disrupt metal ion coordination. Additionally, the role of the cofactor nsp10, which stabilizes the ExoN active site and increases RNA substrate engagement, expands the possibilities of inhibitor design, as disrupting the nsp10/nsp14 interface could synergize with NA-based therapies. Moreover, advancing our understanding of the dynamic interactions of nsp14 with viral RNA and cofactors through integrated structural biology and functional enzymology is critical for the development of next-generation antiviral drugs. These insights would not only accelerate the optimization of existing inhibitors but also enable the design of broad-spectrum inhibitors against CoV, mitigating the risk of future pandemics by targeting conserved viral proteins.

Abbreviations

ATA	Aurintricarboxylic acid
CoVs	Coronaviruses
DMVs	Double-membrane vesicles
dsRNA	Double-stranded RNA
ExoN	Exoribonuclease
GMP	Guanosine monophosphate
Mpro	Main protease
MERS-CoV	Middle East respiratory syndrome coronavirus
N7-MTase	N7-methyltransferase
Nsps	Nonstructural proteins
NAs	Nucleotide analogs
PPI	Protein–protein interaction
RTC	Replication and transcription complex
SAM	S-adenosylmethionine
SARS-CoV	Severe acute respiratory syndrome coronavirus
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
ssRNA	Single-stranded RNA
SEC	Size-exclusion chromatography
ZnF	Zinc finger

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Compliance with Ethical Standards

Conflict of interest Junbo Wang, Yixiao Liu and Zhiyong Lou declare that they have no conflict of interest.

Human and animal rights and informed consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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