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Review

# Recent advances in applying G-quadruplex for SARS-CoV-2 targeting and diagnosis: A review

Li-Yan Zhai [a], Ai-Min Su [a], Jing-Fan Liu [a], Jian-Jin Zhao [a], Xu-Guang Xi [a,b], Xi-Miao Hou [a,*]

[a] College of Life Sciences, Northwest A&F University, Yangling 712100, China
[b] ENS Paris-Saclay, Université Paris-Saclay, CNRS UMR8113, IDA FR3242, Laboratory of Biology and Applied Pharmacology (LBPA), 91190 Gif-sur-Yvette, France

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ABSTRACT

The coronavirus SARS-CoV-2 has caused a health care crisis all over the world since the end of 2019. Although vaccines and neutralizing antibodies have been developed, rapidly emerging variants usually display stronger immune escape ability and can better surpass vaccine protection. Therefore, it is still vital to find proper treatment strategies. To date, antiviral drugs against SARS-CoV-2 have mainly focused on proteases or polymerases. Notably, noncanonical nucleic acid structures called G-quadruplexes (G4s) have been identified in many viruses in recent years, and numerous G4 ligands have been developed. During this pandemic, literature on SARS-CoV-2 G4s is rapidly accumulating. Here, we first summarize the recent progress in the identification of SARS-CoV-2 G4s and their intervention by ligands. We then introduce the potential interacting proteins of SARS-CoV-2 G4s from both the virus and the host that may regulate G4 functions. The innovative strategy to use G4s as a diagnostic tool in SARS-CoV-2 detection is also reviewed. Finally, we discuss some key questions to be addressed in the future.

## 1. Introduction

In late 2019, a novel coronavirus infection was discovered, which soon caused large-scale infection and outbreaks around the world. The WHO has released the official name of the disease as COVID-19, and the virus has been designated severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). SARS-CoV-2 caused a global health care crisis. There have been 599,071,265 confirmed cases of COVID-19, including 6,467,023 deaths reported to WHO as of 4:12 pm CEST, 30 August 2022 (https://covid19.who.int/). Patients present with fever, dry cough, fatigue, dyspnea, mild upper respiratory tract illness, severe viral pneumonia, and even death [1–3], which are similar features in other SARS-CoV infections [4,5]. In addition to SARS-CoV in 2003 [6] and MERS-CoV in 2012 [7], this is the third pandemic in which the coronavirus has caused human social unrest and economic loss during the past 20 years. It is noteworthy that the SARS-CoV-2 genome undergoes persistent and rapid mutations. Since the beginning of 2022, Omicron has started to spread all over the world, displaying a stronger immune escape ability than Delta and better-surpassing vaccine protection. Even now, new variants of Omicron are still emerging. Therefore, it is still vital to find proper therapeutic strategies against SARS-CoV-2.

Although the genomes of the viruses undergo rapid mutations, the secondary structural elements are relatively conserved and therefore can be used as potential antiviral targets. In 1953, Watson and Crick established the B-form double-helical DNA model, opening the era of modern molecular biology [8]. However, non-B-form secondary structures such as *Z*-DNA [9], cruciform DNA [10], triplexes [11], and G-quadruplexes (G4s) [12] have also been found in the genome of many species with essential biological functions, among which G4s have attracted great attention in recent years. As a special type of nucleic acid structure formed by G-rich DNA or RNA sequences, G4s are stacked through the planar G-quartets formed by Hoogsteen bonds and further stabilized by ions (Fig. 1A). These structures can be further classified as intramolecular G4s or intermolecular G4s with different topologies depending on the chain orientation (Fig. 1B). Sequencing of different genomes showed that the location of these putative G-quadruplex-forming sequences (PQSs) is not random. For example, in mammalian cells, telomere G4 consisting of tandemly arranged TTAGGG repeats is the most abundant [13]. In addition, more than 90 % of human DNA replication origins contain G4s, which may be a major determinant involved in the regulation of human cell replication [14]. PQSs are also enriched in proto-oncogenes in humans [15,16]. Overall, the presence of

* Corresponding author.
*E-mail address:* houximiao@nwsuaf.edu.cn (X.-M. Hou).

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G4s is involved in many cellular functions, such as DNA replication, gene expression, and telomere preservation. Interested readers can refer to some excellent reviews [15–18].

Due to their essential biological functions and globular shape similar to proteins, G4s have been developed into anticancer drug targets. While early studies mainly blocked telomere elongation by targeting telomere G4s in cancer cells [19,20], subsequent research has focused on inhibiting the expression of individual oncogenes by ligands, *e.g.*, 3,8,10-trisubstituted isoalloxazines show selective binding to the *c-kit* oncogene promoter G4, thereby inhibiting *c-kit* oncogene expression [21]. Due to the ubiquitous presence of G4s in proto-oncogenes, targeting multiple G4s to simultaneously inhibit the expression of multiple oncogenes is also plausible. The above pieces of evidence suggest that a similar strategy may be applied in antiviral therapy because PQSs are also widely distributed in viral genomes with essential regulatory functions. In particular, a comprehensive analysis of PQSs in the genome of known viruses that can infect humans showed that the occurrence and location of PQSs are features characteristic of each virus class and family [22]. In this context, several recent reviews have systematically summarized the function of viral G4s and the potential of targeting viral G4s in antiviral therapy [23–25].

We noticed that over the past two years since the emergence of COVID-19, many predictions of SARS-CoV-2 PQSs have been made, and some experimentally validated; however, the results seem to vary due to the differences in the prediction software or algorithm used. Therefore, it is necessary to summarize and compare these data. In this review, we first introduce recent studies on SARS-CoV-2 G4s and their targeting by ligands. We searched for the literature related to this topic by the keywords SARS-CoV-2 and G-quadruplex. Most of the studies up to now are included. Then, the potential interacting protein partners of SARS-CoV-2 G4s that may regulate G4 structures are comprehensively reviewed. These results were mainly searched and selected by the keywords SARS-CoV-2, RNA, and interactome. We also discuss the innovative strategy to use G4s as a diagnostic tool in SARS-CoV-2 detection. The related studies were obtained by the keywords SARS-CoV-2, G-quadruplex, detection, or diagnosis. Finally, we present some key questions to be addressed in the future.

## 2. Potential G4s in the SARS-CoV-2 genome and their features

In cells infected with SARS-CoV-2, viral RNA accounts for 65.4 % of the total RNA, which suppresses host gene expression [26]. As a β-coronavirus, SARS-CoV-2 possesses a 29.9-kb-long positive-strand ssRNA genome, which is one of the largest genomes in all RNA viruses. Complex secondary structural features are present in the SARS-CoV-2 genome *in vivo*; interestingly, some are conserved across β-coronaviruses. For instance, Huston et al. reported the complete secondary structures of the SARS-CoV-2 genome using SHAPE-MaP data obtained in living cells, showing many long stretches of short, locally folded stem loops [27]. The protein-coding sections are among the most well-structured regions, suggesting that complex molecular architectures not only appear to be conserved across the β-coronavirus family but also play a functional role in the virus life cycle [27,28]. Manfredonia et al. proved that approximately 10 % of secondary structural features in the SARS-CoV-2 genome show significant covariation and may be relevant to conserved functions. The 3D modeling of these segments further allowed for the identification of putative druggable pockets [29]. Increasing evidence suggests that large-scale structures in SARS-CoV-2 represent important elements in their cellular interactions, which may contribute to their persistence and transmissibility [30]. Meanwhile, disruption of some conserved structures results in inhibition of viral growth [27]. These discoveries together indicate that the secondary structural features of the SARS-CoV-2 genome may play essential roles in the virus life cycle.

As one of the most important noncanonical nucleic acid secondary structures, G4s may be ideal targets in developing innovative drugs against coronaviruses. Therefore, since the very beginning of COVID-19, many studies have focused on PQSs in the SARS-CoV-2 genome. Cui et al. identified 14 PQSs in the positive RNA strand with runs of $G_2N_xG_2N_yG_2N_zG_2$ and loop size from 0 to 12 by the quadruplex forming G-rich sequences mapper (QGRS). Their results further suggested that compared to SARS-CoV, there are fewer PQSs in SARS-CoV-2, which may account for the faster replication rate of SARS-CoV-2 [31]. Panera et al. identified 25 PQSs using QGRS with loop sizes of PQSs from 0 to 36 [32]. Ji et al. not only identified the same 25 results but also found that the PQSs at genome positions 353, 644, 2714, 3467, 8687, 10,261,

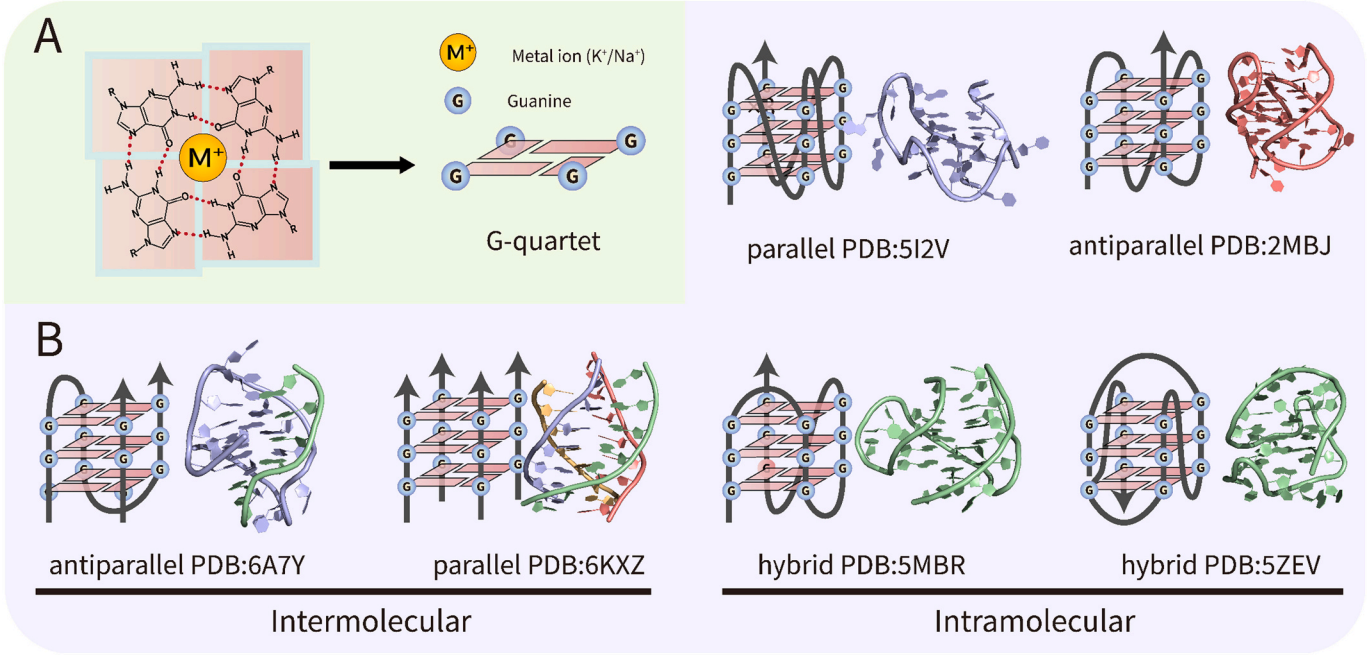


**Fig. 1.** Structures and topologies of G-quadruplexes (G4s). (A) structure of G-quartet. (B) Schematic representation and examples of intramolecular or intermolecular G4s with different topologies.

13,385, 14,947, 15,448, 24,215, 24,268, 26,746, 28,781, and 28,903 are well conserved in the coronavirus family [33]. Later, several studies used other bioinformatics prediction tools (G4CatchAll, pqsfinder, G4Hunter Web, G4screener) and identified some new PQSs, which appeared in the negative strand [34,35]. Researchers also used G4-iM Grinder to analyze the SARS-CoV-2 genome and found a total of 323 PQSs. Although most of them scored poorly, 7 PQSs were scored over 30 with the highest probability of G4 formation, and 4 PQSs were conserved in SARS-coronaviruses and Bat-CoV [36]. Recently, Josué Carvalho et al. analyzed more than 200,000 SARS-CoV-2 genome sequences from five continents and found PQSs at positions 1574, 13,385, 24,215, 24,268, and 25,197 with a high conservation level above 98 %. Besides, PQS at position 28,903 in the N protein-coding sequence revealed 90.82 % conservation [37]. The potential roles played by the RNA G4s in the SARS-CoV-2 life cycle are illustrated in Fig. 2, including translation of Nsps and structural proteins, RNA transcription, RNA replication, and packaging of the genome.

In addition to bioinformatic prediction, some PQSs have been confirmed by circular dichroism (CD) spectroscopy analysis, nuclear magnetic resonance (NMR), fluorescence turn-on assays, multiple other biophysical techniques, and molecular biology assays [31–33,36,38,39]. In particular, the first calculated G4 structure of RG-1 at position 28,903 was recently reported by a multiscale approach combining quantum and classical molecular modeling [40]. We summarize the PQSs that have been predicted or verified in Table 1. Importantly, all of them contain two G-quartets once folded. The conserved PQSs among coronaviruses are shown in Table 2. Due to differences in prediction software or algorithms, there are some inconsistencies among these results. Therefore, these data will require rigorous experimental verification.

## 3. Targeting RNA G4s in SARS-CoV-2 by small-molecule compounds and natural products

Recently, Zhao et al. used multiple approaches, such as fluorescence turn-on assays, CD, NMR, and fluorescence resonance energy transfer (FRET), to show that RG-1 at the coding region of the SARS-CoV-2 N protein, indeed forms a G4 structure *in vitro* [38]. In the following experiment, for the first time, PQS in the SARS-CoV-2 genome was verified to fold into stable unimolecular G4 structures in live cells. It is worth noting that G4-specific compounds, such as PDP (pyridostatin) (Fig. 3A), a bisquinolinium derivative that inhibited HCV replication in cells with a half-maximum inhibitory concentration ($IC_{50}$) of 1.2 μM [41], can stabilize RG-1 and significantly reduce the expression levels of the N protein not only *in vitro* but also *in vivo* (Fig. 2) [38]. For instance, the HeLa cells pre-transfected with Cy5-labeled RG-1-WT and RG-1-Mut were treated with 4 μM PDP. As a result, the colocalization of RG-1-WT and G4 antibody was significantly increased, but not for RG-1-Mut, suggesting the promotion of G4 structure formation by PDP. The authors further found that 4 μM PDP treatment can inhibit the protein expression of Flag-N-RG-1-WT in both HeLa and HEK293T cells to ~1/5 of the control without PDP (~1.0 to ~0.2). However, the protein expression of Flag-N-RG-1-Mut was unchanged upon PDP treatment. These results indicate that PDP can inhibit N protein expression by targeting RG-1. The related equilibrium dissociation constant $K_D$ and $IC_{50}$ values remain to be determined in the future.

Classical molecular dynamics trajectories (MD) are consistent with the experimental data in confirming the folding of RG-1 into a parallel G4 conformation composed of two rigid tetrads and a flexible peripheral loop (Fig. 4A). Moreover, Miclot et al. further obtained docking

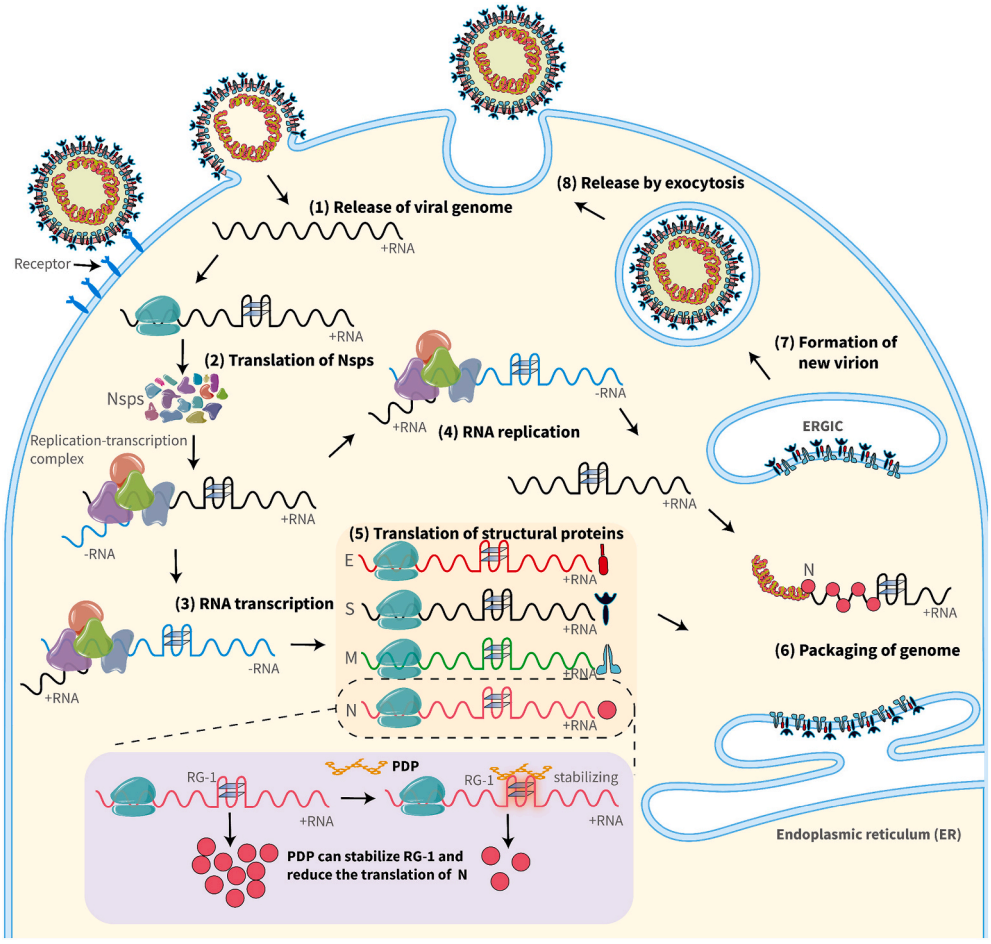


**Fig. 2.** Possible roles of RNA G4s in the SARS-CoV-2 life cycle. Different steps of viral infection are illustrated. (1) Release of the viral genome; (2) Translation of Nsps; (3) RNA transcription; (4) RNA replication; (5) Translation of structural proteins; (6) Packaging of the genome; (7) Formation of new virion; (8) Release by exocytosis. Virus G4s may be involved in regulating the efficiency of steps 2–6 and may become a target for antiviral therapy. For instance, the G4-specific ligand PDP can stabilize RG-1 (at genome position 28,903) and reduce the translation of the nucleocapsid (N) protein.

**Table 1**
PQSs in the SARS-CoV-2 genome.

| | G4 position | Gene | Sequence | QGRS mapper Score | G4Hunter score | G4-iM grinder Score | Reference of prediction | Confirmed by reference |
|---|---|---|---|---|---|---|---|---|
| 1 | +236 | 5′-UTR | GGUUUCGUCCGGGUGUGACCGAAAGGUAAGAUGG | | | | [31] | |
| 2 | +353 | Nsp1 | GGCUUUGGAGACUCCGUGGAGGAGG | 16 | 0.64 | | [31–35] | [31] |
| 3 | +359 | Nsp1 | GGAGACUCCGUGGAGGAGG | | | 30 | [34,36] | |
| 4 | +370 | Nsp1 | GGAGGAGGUCUUAUCAGAGG | | | 30 | [36] | |
| 5 | +545 | Nsp1 | GGCAUUCAGUACGGUCGUAGUGGUGAGACACUUGG | | | | [31] | |
| 6 | +644 | Nsp1 | GGUAAUAAAGGAGCUGGUGG | 15 | 0.8 | 30 | [31–36] | [31,39] |
| 7 | +1463 | Nsp2 | GGUGGUCGCACUAUUGCCUUUGGAGG | 6 | 0.423 | | [32,33,35] | |
| 8 | +1574 | Nsp2 | GGUGUUGUUGGAGAAGGUUCCGAAGG | 18 | 0.615 | | [31–33,35,37] | |
| 9 | +2714 | Nsp2 | GGCGGUGCACCAACAAAGGUUACUUUUGG | 10 | 0.31 | | [31–33,35] | |
| 10 | +3467 | Nsp3 | GGAGGAGGUGUUGCAGG | 15 | 1 | 34 | [31–36] | [36,39] |
| 11 | +4162 | Nsp3 | GGUUAUACCUACUAAAAAGGCUGGUGG | 6 | 0.37 | | [32,33,35] | |
| 12 | +4255 | Nsp3 | GGGUCAGGGUUUAAAUGGUUACACUGUAGAGGAGG | | | 31 | [31,36] | |
| 13 | +4261 | Nsp3 | GGGUUUAAAUGGUUACACUGUAGAGGAGG | 10 | 0.933 | | [32–35] | |
| 14 | +4262 | Nsp3 | GGUUUAAAUGGUUACACUGUAGAGGAGG | 10 | | | [31,34] | |
| 15 | +5036 | Nsp3 | GGACAACAGUUUGGUCCAACUUAUUUGGAUGG | | | | [31] | |
| 16 | +8687 | Nsp4 | GGAUACAAGGCUAUUGAUGGUGG | 14 | 0.652 | | [31–35] | |
| 17 | +10,058 | Nsp5 | GGUUUUAGAAAAAUGGCAUUCCCAUCUGGUAAAGUUGAGG | | | | [31] | |
| 18 | +10,255 | Nsp5 | GGUACAGGCUGGUAAUGUUCAACUCAGG | | | | [34] | |
| 19 | +10,261 | Nsp5 | GGCUGGUAAUGUUCAACUCAGGGUUAUUGG | 9 | 0.6 | | [32–35] | |
| 20 | +13,385 | Nsp10 | GGUAUGUGGAAAGGUUAUGG | 19 | 1.048 | 31 | [31–37] | [31,33,38] |
| 21 | +14,947 | Nsp12 | GGUUUUCCAUUUAAUAAAUGGGGUAAGG | 4 | 0.714 | | [32,33,35] | |
| 22 | +15,208 | Nsp12 | GGAACAAGCAAAUUCUAUGGUGGUUGG | 6 | 0.678 | | [32,33,35] | |
| 23 | +15,448 | Nsp12 | GGCGGUUCACUAUAUGUUAAACCAGGUGG | 3 | 0.345 | | [32,33,35] | |
| 24 | +18,296 | Nsp14 | GGAUUGGCUUCGAUGUCGAGGGG | 9 | 1.043 | | [31–35] | |
| 25 | +20,869 | Nsp16 | GGUGCUGGUUCUGAUAAAGGAGUUGCACCAGG | | | | [31] | |
| 26 | +22,316 | S | GGUGAUUCUUCUUCAGGUUGGACAGCUGG | 10 | 0.448 | | [32,33,35] | |
| 27 | +24,215 | S | GGUUGGACCUUUGGUGCAGG | 17 | 0.6 | | [31–33,35,37] | [31] |
| 28 | +24,268 | S | GGCUUAUAGGUUUAAUGGUAUUGG | 19 | 0.625 | | [31–35,37] | [31,33] |
| 29 | +25,197 | S | GGCCAUGGUACAUUUGGCUAGG | 17 | 0.455 | | [31–35,37] | [31] |
| 30 | +25,951 | ORF3a | GGUGGUUAUACUGAAAAAUGGGAAUCUGG | 8 | 0.69 | | [32–35] | |
| 31 | +26,746 | M | GGAUCACCGGUGGAAUUGCUAUCGCAAUGG | 7 | 0.333 | | [32–35] | |
| 32 | +28,613 | N | GGAACUGGGCCAGAAGCUGGACUUCCCUAUGG | | | | [31] | |
| 33 | +28,781 | N | GGCUUCUACGCAGAAGGGAGCAGAGGCGG | 9 | 0.655 | | [32,33,35] | |
| 34 | +28,903 | N | GGCUGGCAAUGGCGG | 18 | 0.867 | 34 | [31–33,35–37] | [31,36,38,39] |
| 35 | +29,123 | N | GGAAAUUUUGGGGACCAGG | 14 | 1.053 | | [31–33,35] | |
| 36 | +29,234 | N | GGCAUGGAAGUCACACCUUCGGGAACGUGG | 11 | 0.467 | | [32,33,35] | |
| 37 | +29,254 | N | GGGAACGUGGUUGACCUACACAGGUGCCAUCAAAUUGG | | | | [31] | |
| 38 | −165 | | GGCCUCGGUGAAAAUGUGGUGG | 13 | 0.591 | | [31,35] | |
| 39 | −1591 | | GGGGUGCAUUUCGCUGAUUUUGGGG | | 1.280 | | [35] | |
| 40 | −2987 | | GGUCUGGUCAGAAUAGUGCCAUGGAGUGG | 9 | 0.483 | | [34,35] | |
| 41 | −6822 | | GGUUGGUAACCAACACCAUUAGUGGGUUGG | 6 | 0.433 | | [35] | |
| 42 | −11,440 | | GGCGGUGGUUUAGCACUAACUCUGG | 7 | 0.48 | | [35] | |
| 43 | −13,136 | | GGUUAAGUGGUGGUCUAGG | 16 | 0.842 | | [31,35] | |
| 44 | −13,963 | | GGAUCUGGGUAAGGAAGG | 19 | 1.111 | | [31,34,35] | [39] |
| 45 | −16,623 | | GGAUUUGGAUGAUCUAUGUGGCAACGG | 14 | 0.556 | | [31,35] | |
| 46 | −19,865 | | GGUGAUAGAGGUUUGUGGUGGG | | | | [34] | |
| 47 | −19,865 | | GGUGAUAGAGGUUUGUGGUGGUUGG | 19 | 0.92 | | [31,34,35] | |
| 48 | −19,874 | | GGUUUGUGGUGGUUGG | | | | [34] | |
| 49 | −23,877 | | GGAUAUGGUUGGUUUGG | 19 | 0.941 | | [31,34,35] | [39] |
| 50 | −25,003 | | GGUGGAAUGUGGUAGG | 17 | 1.063 | | [31,34,35] | |
| 51 | −27,432 | | GGGGCUUUUAGAGGCAUGAGUAGG | 13 | 1.042 | | [31,34,35] | |
| 52 | −29,867 | | GGUUGGUUUGUUACCUGGGAAGG | 13 | 0.783 | | [31,34,35] | |

complexes of PDP and CX-3543 (also referred to as quarfloxin, a G4 ligand derived from fluoroquinolones) (Fig. 3B) with RG-1 (Fig. 4B-C). The binding takes place by π-π stacking of the ligands on top of the G-quartet, and it is mostly driven by dispersion and hydrophobic interactions. There is no significant energetic barrier or steric hindrance, which greatly facilitates the recruitment of the targeting ligands. Therefore, both G4 ligands can potentially be seen as valuable compounds to stabilize G4 arrangements, particularly the parallel conformation of RG-1 [40].

Researchers have been striving to find effective G4-specific ligands to target G4s in SARS-CoV-2. Berberine is a planar molecule with an extended π-delocalized system (Fig. 3C), which can interact with G-quartets of G4 *via* π-π stacking interactions [42]. In addition, Berberine is not fluorescent in the aqueous solution, whereas a strong fluorescence increase can be observed upon binding to G4. As a common folk medicine used for hundreds of years in China, berberine has shown antiviral, anti-allergic, and anti-inflammatory properties [43]. Quite recently, the binding properties of berberine to RG-1 were characterized [44]. Oliva et al. designed a titration experiment that kept the concentration of RG-1 constant, while the concentration of berberine was varied. The reported data indicate that berberine can interact with RG-1 with binding constants of $2.8 \pm 0.3 \times 10^4\ M^{-1}$ in NaCl and $1.48 \pm 0.3 \times 10^4\ M^{-1}$ in KCl. Further results showed that two berberine molecules are bound to one RG-1. In addition, RG-1 adopts a parallel conformation in both the ligand-free and ligand-bound states. All the above evidence suggests that, as therapeutic targets, SARS-CoV-2 G4s may provide a promising direction for humans to fight against SARS-CoV-2 (Table 3).

In addition to the G4s in the SARS-CoV-2 genome, the host G4s that regulate the expression of crucial proteins for virus entry can also be targeted for antiviral therapy. For instance, the transmembrane serine

**Table 2**
Conserved PQSs in the SARS-CoV-2 genome.

| | G4 position | Gene | Sequence | Found in other members of the Coronaviridae family | Reference |
|---|---|---|---|---|---|
| 1 | +353 | Nsp1 | GGCUUUGGAGACUCCGUGGAGGAGG | SARS-CoV<br>Bat-CoV | [31,33] |
| 2 | +644 | Nsp1 | GGUAAUAAAGGAGCUGGUGG | SARS-CoV<br>Bat-CoV<br>BtRl-BetaCoV<br>BtRs-BetaCoV<br>BtRf-BetaCoV<br>Rhinolophus-affinis-coronavirus | [31,33] |
| 3 | +2714 | Nsp2 | GGCGGUGCACCAACAAAGGUUACUUUUGG | Bat-CoV | [33] |
| 4 | +3467 | Nsp3 | GGAGGAGGUGUUGCAGG | BtRt-BetaCoV | [33] |
| 5 | +8687 | Nsp4 | GGAUACAAGGCUAUUGAUGGUGG | Bat-CoV | [33] |
| 6 | +10,261 | Nsp5 | GGCUGGUAAUGUUCAACUCAGGGUUAUUGG | Bat-CoV | [33] |
| 7 | +13,385 | Nsp10 | GGUAUGUGGAAAGGUUAUGG | SARS-CoV<br>Bat-CoV<br>Rhinolophus-affinis-coronavirus | [31,33,36] |
| 8 | +14,947 | Nsp12 | GGUUUUCCAUUUAAUAAAUGGGGUAAGG | SARS-CoV<br>Bat-CoV<br>BtRs-BetaCoV<br>Rhinolophus-affinis-coronavirus | [33] |
| 9 | +15,448 | Nsp12 | GGCGGUUCACUAUAUGUUAAACCAGGUGG | SARS-CoV<br>Bat-CoV<br>BtRl-BetaCoV<br>BtRs-BetaCoV<br>Rhinolophus-affinis-coronavirus | [33] |
| 10 | +24,215 | S | GGUUGGACCUUUGGUGCAGG | SARS-CoV<br>Bat CoV | [31,33] |
| 11 | +24,268 | S | GGCUUAUAGGUUUAAUGGUAUUGG | SARS-CoV<br>Bat-CoV<br>BtRf-BetaCoV<br>Rhinolophus-affinis-coronavirus | [31,33] |
| 12 | +25,197 | S | GGCCAUGGUACAUUUGGCUAGG | SARS-CoV<br>Bat-CoV | [31] |
| 13 | +26,746 | M | GGAUCACCGGUGGAAUUGCUAUCGCAAUGG | Bat-CoV<br>SARS-CoV | [33] |
| 14 | +28,781 | N | GGCUUCUACGCAGAAGGGAGCAGAGGCGG | Bat-CoV<br>BtRs-BetaCoV<br>Rhinolophus-affinis-coronavirus | [33] |
| 15 | +28,903 | N | GGCUGGCAAUGGCGG | SARS-CoV<br>Bat-CoV | [31,33] |

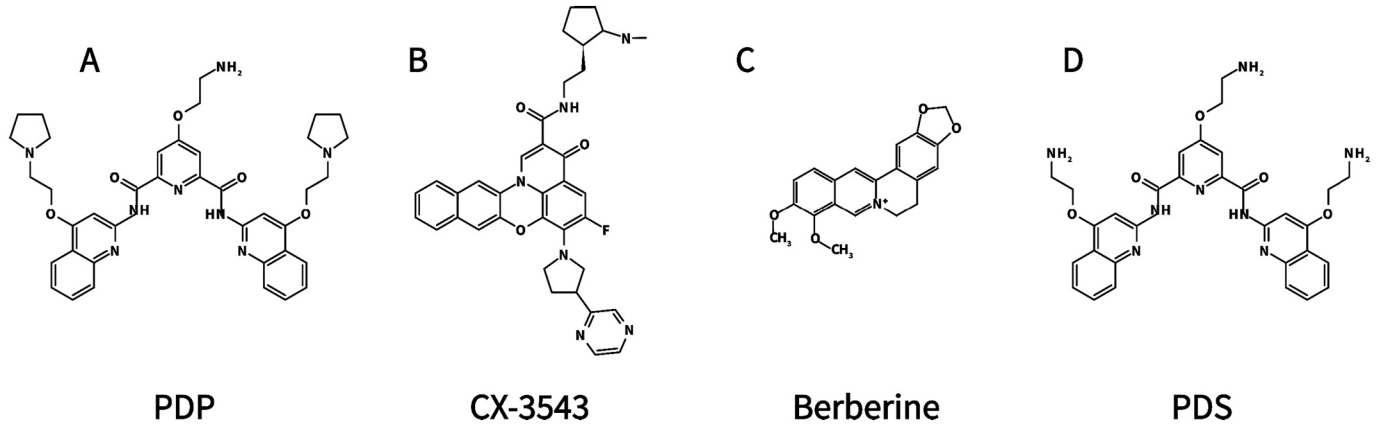


**Fig. 3.** Chemical structures of (A) PDP, (B) CX-3543, (C) Berberine, and (D) PDS.

protease TMPRSS2 can cleave the hemagglutinin of many subtypes of influenza virus and the spike proteins of coronavirus [45]. In particular, TMPRSS2 has been identified as one of the essential host determinants for SARS-CoV-2 infection [46]. In the promoter of the human *Tmprss2* gene, there is a G-rich tract capable of forming a G4 structure in the presence of $K^+$, significantly affecting the transcription level of *Tmprss2* [45]. Moreover, in the open reading frame, an RNA G4 motif appears, inhibiting *Tmprss2* translation and SARS-CoV-2 entry in cells. The G4-stabilizer PDS (pyridostatin) (Fig. 3D), which promoted the folding of telomeric G4 with a $K_D$ of 490 ± 80 nM [47], also binds RNA G4 named PQS-675 in *Tmprss2* with the $K_D$ of 604.9 ± 2.84 nM. Furthermore, PDS can attenuate the infection of SARS-CoV-2 pseudoviruses in human lung cells, as shown by the fact that the entry efficiency with 50 μM PDS treatment is only about 10 % of the control. The half-maximal effective concentration ($EC_{50}$) is between 2 and 5 μM. In addition to PDS, G4-stabilizer cPDS and TMPyP4 also significantly reduce pseudovirus entry efficiency with the $EC_{50}$ around 50 μM and 20 μM, respectively. In mouse models, researchers found that PDS-treated mice have a significantly decreased entry of pseudoviruses (~20 %) compared with saline-treated mice. Immunohistochemical, ELISA, and western blot

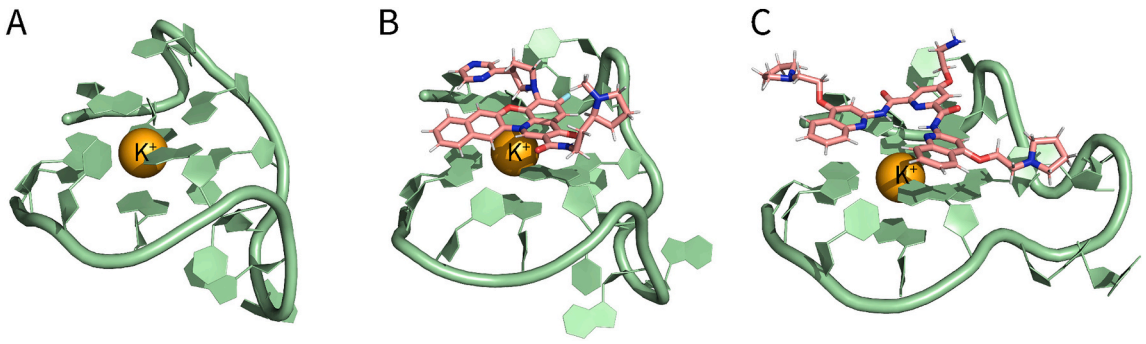


**Fig. 4.** Structure of RG-1 and its molecular docking with ligands. (A) Calculated structure of RG-1. (B) Molecular docking of RG-1 and CX-3543. (C) Molecular docking of RG-1 and PDP. (Structural data from the study by Miclot et al. [40]).

**Table 3**
The small-molecule compound used in G4 targeting for SARS-CoV-2 treatment.

| Ligand | targeting G4 | G4 origin | G4 sequence | Effect | Reference |
|---|---|---|---|---|---|
| PDP | RG-1 | SARS-CoV-2 N protein coding sequence | **GGCUGGCAAUGGCGG** | It stabilizes the RG-1 and significantly reduces the expression levels of the SARS-CoV-2 N protein. | [38] |
| Berberine | RG-1 | SARS-CoV-2 N protein coding sequence | **GGCUGGCAAUGGCGG** | It interacts with RG-1 with binding constants of $2.8 \pm 0.3 \times 10^4$ $M^{-1}$ in NaCl, and $1.48 \pm 0.3 \times 10^4$ $M^{-1}$ in KCl | [44] |
| PDS | PQS-675 | Host TMPRSS2 coding sequence | **GGGCGGGCGGCCUGCAGGGACAUGGG** | It inhibits SARS-CoV-2 entry in cells and hinders SARS-CoV-2 infection *in vivo*. | [46] |

experiments further showed that PDS administration led to a decrease in TMPRSS2 protein levels in the lungs [46]. These results together indicated that *Tmprss2* RNA G4 is a potential target for SARS-CoV-2 inhibition (Table 3).

## 4. Regulation of SARS-CoV-2 G4s by proteins

As shown in Fig. 5, the SARS-CoV-2 genome contains 14 open reading frames (ORFs). ORF1a/ORF1b encodes nonstructural proteins (NSPs) 1–16, most of which are enzymes related to virus replication and transcription. At the 3′ end, the genome encodes 4 structural proteins, spike (S), envelope (E), membrane (M), and nucleocapsid (N), which play vital roles in viral structure integrity. The genome also encodes 9 accessory factors [48]. It is worth noting that some of these proteins have been reported to interact with or have the potential to interact with RNA G4s.

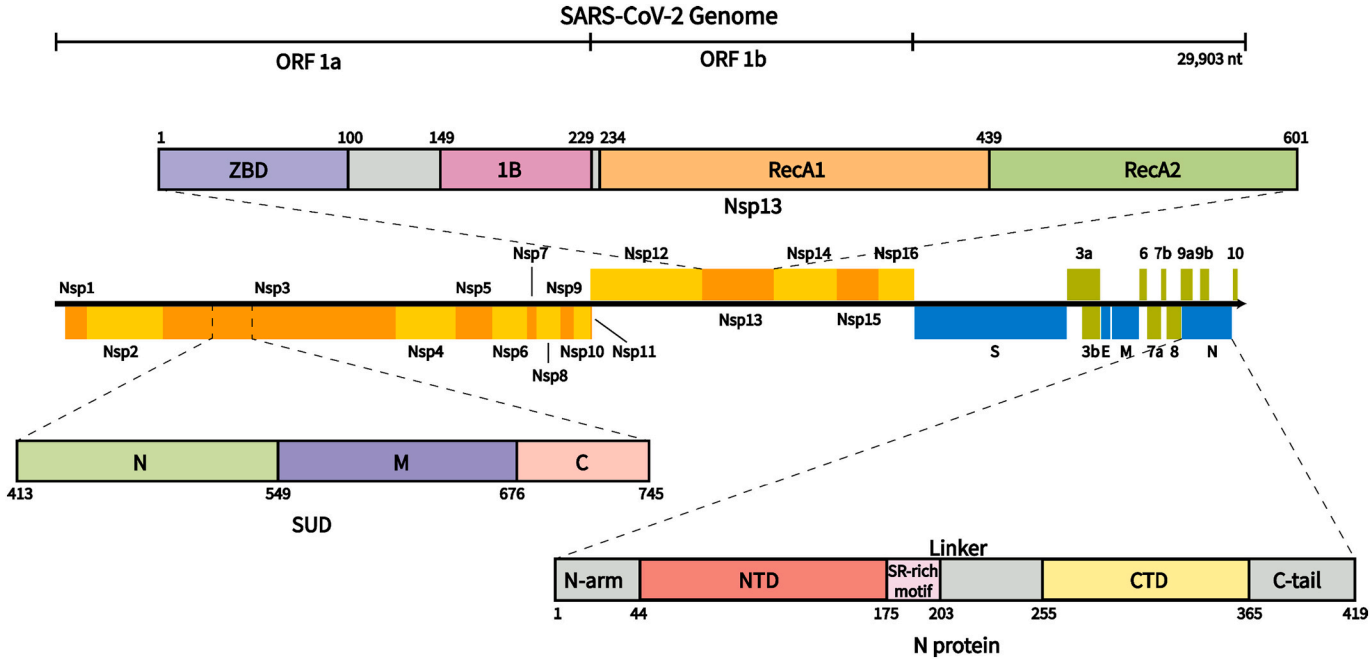


**Fig. 5.** SARS-CoV-2 genome organization and the schematic domain structure of SUD, Nsp13, and N protein. ORF1a/ORF1b encodes Nsps 1–16; at the 3′ end, the genome encodes 4 structural proteins, spike (S), envelope (E), membrane (M), nucleocapsid (N), and 9 accessory factors. SUD contains SUD-N, SUD-M, and SUD-C domains; Nsp13 contains a zinc-binding domain (ZBD), 1B domain, two RecA-like domains, and a stalk domain; N protein contains two structural domains, N-NTD, N-CTD, and three intrinsically disordered regions.

### 4.1. SUD in Nsp3

Nsp3 is the largest Nsp in SARS coronaviruses, consisting of 1922 amino acid residues [49,50]. As part of the SARS coronavirus replicase/transcriptase complex, Nsp3 plays an important role in the formation and activity of the viral replication/transcription complex (RTC) [51]. Nsp3 consists of at least seven domains, one of which is the SARS-Unique Domain (SUD) (Fig. 5). X-ray crystallography and NMR spectroscopy indicate that the SUD comprises two macrodomains and one frataxin-like domain, named SUD-N, SUD-M, and SUD-C, respectively. SUD-C is not stable in full-length SUD; thus, SUD-N and SUD-M constitute the core domain. The full-length SUD has 338 amino acid residues, while SUD-NM (SUD-core) consists of 264 amino acid residues. The 3D structure shows that SARS-CoV SUD-NM is in a head-to-tail dimer form (Fig. 6A) [52–54]. It is worth noting that SUD is not conserved among coronaviruses that can infect humans. The SUD was only found in SARS-CoV and SARS-CoV-2, with an amino acid identity of 75 % and similarity of 95 % [55]; however, it was incomplete in MERS-CoV and absent in other less pathogenic coronaviruses [34,55,56]. Therefore, the SUD may play an important role in the strong pathogenicity of SARS coronaviruses.

Tan et al. showed that the SARS-CoV SUD or its subdomains SUD-N and SUD-M can bind oligo(G) stretches known to form G4s, and the affinity was enhanced by the addition of $K^+$, revealing the SUD as a G4-binding protein for the first time [52,53]. More recently, Lavigne et al. confirmed that the SUD is also present in the Nsp3 of SARS-CoV-2. The authors further showed that the SARS-CoV-2 SUD can interact with both DNA and RNA G4s. Because this interaction can be disrupted by G4 ligands, the authors then proposed that inhibitors of their interactions may be used as potential antiviral compounds [55]. Molecular simulations further showed that RNA G4 can favor and stabilize the dimerization of the SUD, which may be related to its biological role in assisting the SARS virus to bypass the protective response of the host [57]. However, previous studies have not clarified the effect of SUD binding on G4s. Whether the SUD promotes G4 formation, stabilizes, or disrupts G4 structures is still unknown.

At the functional level, SUD/G4 interaction first has a significant influence on the activity of SARS-CoV RTC, in which the SUD-M domain is crucial while SUD-N is dispensable [58]. This observation may be consistent with the findings of Tan et al., who showed that mutations of selected lysine residues on the surface of SUD-N led to a reduction in G4 binding, whereas mutations in SUD-M abolished it [53]. Importantly, these amino acids are conserved in SARS-CoV-2 [55], suggesting that the replication of the SARS-CoV-2 genome may also require the SUD, especially SUD-M, to interact with G4s. Second, as Nsp3 has not been reported to enter the nucleus, the host targets of the SUD are most likely RNA G4s. Many RNA G4s reside in both the non-translated and translated regions of mRNAs coding for host cell proteins involved in apoptosis or signal transduction [51,58–63]. These proteins can induce controlled cellular death of infected cells, thereby slowing or preventing infection, or promoting cell survival by producing antiviral cytokines [62]. However, the binding of the viral SUD to mRNA G4s necessary for the production of the above signaling factors may impair the apoptosis/survival response pathways and then allow massive cell infection [57]. Altogether, the SUD may regulate both viral replication and the host immune response by binding to RNA G4s.

In addition to G4s, the SUD can also directly interact with host cell proteins. For instance, the interaction between the SUD and $PL^{pro}$ increases the stability of RCHY1 and augments RCHY1-mediated p53 ubiquitination. Consequently, p53 degradation is upregulated, thereby promoting viral replication [64]. A recent study showed that both SARS-CoV and SARS-CoV-2 SUDs interact with human poly(A)-binding protein (PAPB)-interacting protein 1 (Paip1) [56]. This interaction enhances SARS-CoV RNA translation but not the translation of host proteins, indicating that the virus takes advantage of the host translation machinery for its benefit [56]. Lavigne et al. believed that the SUD/Paip1 interaction may be involved in the selective translation regulation of G4-containing mRNAs in infected cells [55]. SUDs combined with G4s may recruit Paip1 and interfere positively or negatively with the recruitment of other translation factors, given that the SUD binding site to Paip1 is different from that to G4 [55]. Therefore, the SUD/Paip1 interaction may offer a new antiviral target for SARS-CoV-2. In addition, the SARS-CoV SUD modulated NLRP3 inflammasome-dependent CXCL10-mediated pulmonary inflammation [65]. However, the molecular mechanism has not yet been characterized. Perhaps there are other undiscovered SUD-interacting proteins in the host cell, and more work needs to be done to clarify the biological functions of the SUD, in particular, SUD-G4 interactions. Altogether, we think that targeting the SUD seems reasonable given its specificity in coronaviruses and its high G4 binding affinity.

### 4.2. Nsp13

Coronavirus Nsp13 is a superfamily 1 helicase (SF1) with NTPase activity [66,67] that can unwind both RNA and DNA substrates in the 5′-to-3′ direction [68]. Recently, SARS-CoV-2 Nsp13 was also shown to have the same activities [66]. Nsp13 is very conserved in evolution. The SARS-CoV-2 Nsp13 contains 601 aa, with only one amino acid substitution to SARS-CoV (V570 in SARS-CoV-2 to I570 in SARS-CoV) [69]. As shown in Fig. 6B, the pyramid-shaped SARS-CoV-2 Nsp13 contains two typical SF1 helicase RecA domains, an N-terminal zinc-binding domain (ZBD), a stalk, and a 1B domain specific to coronavirus helicases [70,71]. Six key residues, K288, S289, D374, E375, Q404, and R567, which cluster in the base cleft between the RecA1 and RecA2 domains, are involved in NTP hydrolysis [70].

In the host cell, Nsp13 localizes to the endoplasmic reticulum or an endoplasmic reticulum-derived membrane compartment, suggesting that it is likely the core of the coronavirus replication machinery [72]. This is consistent with the structure of a stable Nsp13-replication-transcription complex determined by cryo-electron microscopy (cryo-

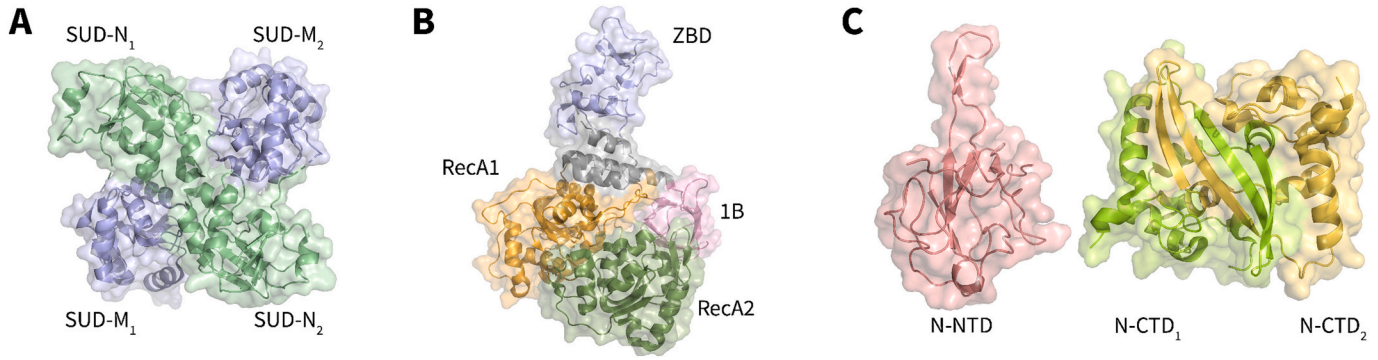


**Fig. 6.** Crystal structures of (A) SARS-CoV SUD dimer (PDB: 2W2G), (B) SARS-CoV-2 Nsp13 (PDB: 6ZSL), (C) SARS-CoV-2 N protein (PDB: 7CDZ/7CE0).

EM) to 3.5-Å nominal resolution [69]. Further study demonstrated that Nsp13 could act in the 5′-3′ direction on tRNA to disrupt stable RNA secondary structures or downstream RNA binding proteins, both of which could be significant impediments to RNA elongation [69]. In addition to its roles in replication, Nsp13 binds and inhibits TBK1 phosphorylation, resulting in decreased IRF3 activation and IFN-β production. The above evidence suggests that Nsp13 is also involved in viral evasion of immune responses [73].

Shum et al. used exponential enrichment (SELEX) as an *in vitro* selection strategy to isolate DNA sequences that have a high affinity for Nsp13. They found that most of the sequences were guanosine rich and likely to fold into G4 structures [74]. This may indicate a binding preference of Nsp13 for G4. Recently, Ji et al. used microscale thermophoresis (MST) to measure the interaction between Nsp13 and G4s at positions 13,385 and 24,268 within the SARS-CoV-2 genome. Their results showed that Nsp13 interacts with G4 at 13385 and G4 at 24268 with $K_D$ values of 0.79 ± 0.14 and 0.37 ± 0.08 μM, respectively. Subsequently, molecular docking showed that the guanine bases in G4s deviated significantly from their expected placement in both cases. These guanine bases are predicted to be flipped away from the G-quartets, thus reflecting the unfolding of SARS-CoV-2 G4 structures by Nsp13 [33].

It is worth noting that since the outbreak of SARS-CoV in 2003, research on the development of inhibitors of Nsp13 has emerged. Bananins [75], dihydroxychromone derivatives [76], SSYA10–001, and a 1,2,4 triazole [77] have all been reported to be potent inhibitors of SARS-CoV Nsp13. Recently, lumacaftor and cepharanthine were shown to inhibit SARS-CoV-2 Nsp13 ATPase activity [78]. Natural flavonoids were also identified as selective inhibitors of SARS-CoV-2 Nsp13 [79]. Together, these pieces of evidence reflect that targeting Nsp13 could be one of the major areas of anti-SARS-CoV-2 research.

### 4.3. N protein

Nucleocapsid (N) protein can bind to the SARS-CoV-2 genome and package the RNA into the ribonucleoprotein (RNP) complex, which plays a vital role during viral self-assembly [80]. As shown in Fig. 5, β-coronavirus N proteins share a common overall domain organization, including the RNA-binding domain (N-NTD) and dimerization domain (N-CTD) that are separated by short regions with a high predicted disorder [81,82]. The SARS-CoV-2 N protein contains 419 aa [48]. Huang et al. solved the 3D structure of the N-terminal domain of the SARS-CoV N protein, which consists of a five-stranded β-sheet with a folding topology distinct from other RNA-binding proteins. The ssRNA binds to the protein surface at the junction between a flexible positively charged beta-hairpin and the core structure [83]. Recently, Peng et al. solved the crystal structures of SARS-CoV-2 N-NTD and N-CTD at 1.8 Å and 1.5 Å resolution, respectively (Fig. 6C). Both structures show conserved features from other coronavirus N proteins. The N-NTD presents a right-handed fist shape and consists of a four-stranded antiparallel-sheet core subdomain. The protruding loops are positively charged, providing a putative site for RNA binding [82]. The N-CTD displays a compact, intertwined dimer similar to that of related coronaviruses, including SARS-CoV [81,84].

The N protein can recognize packaging signals to mediate coronavirus genomic RNA packaging into virions [85,86]. In addition, many other RNA processing, RNA metabolism, and transcriptional regulatory proteins have been identified as SARS-CoV-2 N protein-interacting partners, which suggests that the N protein may be involved in multiple viral RNA processes [87]. Interestingly, several recent studies revealed that SARS-CoV-2 N protein and RNA underwent liquid-liquid phase separation depending on the length and concentration of ssRNA [88,89]. Moreover, the SARS-CoV-2 N protein can be recruited to phase-separated assemblies formed by human proteins, providing a potential mechanism for the role of the N protein in SARS-CoV-2 viral genome packaging [89]. In addition to genome packaging, SARS-CoV N protein can inhibit S phase progression in mammalian cells [90] and antagonize immune regulation by targeting the initial step of the IFN-β induction pathway [91]. SARS-CoV-2 N protein can directly interact with NLRP3 to promote NLRP3 inflammasome activation, leading to aggravation of lung injury [92]. In addition, as viral suppressors of RNAi, both SARS-CoV-2 and SARS-CoV N proteins antagonize the RNAi pathway to protect viral RNA in cells [93,94]. These findings all indicate the importance of the N protein and its perspectives for drug design as well as vaccine development.

In infected cells, SARS-CoV-2 and SARS-CoV N proteins localize exclusively in the cytoplasm [95,96]. During viral self-assembly, RNA needs to be packaged by N protein, while during viral infection, RNA needs to be released (Fig. 2). Therefore, the N protein may play an important role in remodeling and maintaining the RNA structures in the virus. In this regard, HIV-1 nucleocapsid protein (NCp7) was reported to readily destabilize and unfold DNA G4 [97]. Butovskaya et al. further demonstrated that NCp7 binds and unfolds HIV-1 RNA G4s and promotes DNA/RNA duplex formation, allowing reverse transcription to proceed [98] (Fig. 7). These studies also suggest a possible role of the SARS-CoV-2 N in regulating RNA G4; however, this speculation still needs to be verified by experiments.

During viral infection, some host helicases, especially members of the DEAD-box family act as proviral factors [99]. Recently, Squeglia et al. showed that hijacking of the DDX helicase by SARS-CoV-2 is expected to act as a proviral process by enhancing key steps in the viral life cycle [100]. Ciccosanti et al. further identified DDX3X and DHX9 as the interactome of the SARS-CoV-2 N protein and suggested that the N protein recruits DDX3X to the replication complex for virus production [101]. Researchers have also directly observed the association of DDX21 with the SARS-CoV-2 N protein, but how this interaction affects viral replication and infection is still unknown [102]. As DDX3X [103], DDX21 [104], and DHX9 [105] have all been shown to bind RNA G4s, these studies may implicate the interplay of N proteins with G4s, either through their own binding activity or through the hijacking of helicases (Fig. 7).

### 4.4. Cellular G4-binding proteins

Coronaviruses need host factors to facilitate their replication [106,107], and SARS-CoV-2 has developed multiple strategies to interact with a complex network of host antiviral pathways [108,109]. Recently, several studies have focused on the interactome between host proteins and the SARS-CoV-2 genome. For instance, Lee et al. identified 109 host factors that directly bind to SARS-CoV-2 RNA, in which they delineated 17 antiviral and 8 proviral RNA binding proteins (RBPs) hijacked by the virus [110]. Kamel et al. showed that SARS-CoV-2 infection profoundly remodels the cellular RNA-bound proteome and identified the proteins directly interacting with viral RNA, uncovering dozens of cellular RNA-binding proteins and 6 viral proteins [111]. Schmidt et al. identified up to 104 human proteins that directly and specifically bind to SARS-CoV-2 RNA in infected cells, 38 of which are unique SARS-CoV-2 RNA binders [112]. Flynn et al. identified 309 host proteins that bind SARS-CoV-2 RNA during infection [113]. Most of them serve as antiviral factors with a functional impact on host cell survival, suggesting that the initial response of the host to viral infection is to identify viral RNA and control the viral life cycle [113]. Taken together, a large number of cellular proteins bind to SARS-CoV-2 RNA in infected cells, highlighting viral RNA as a hub for complex host-virus interactions.

It is worth noting that some of the RBPs, such as nucleolin, hnRNPs, and helicases, that appeared in the SARS-CoV-2 genome interactome have already been reported to interact with G4s in other viruses (Fig. 7). For instance, nucleolin interacts with RNA G4 formed in EBNA1 mRNA to restrict EBNA1 expression in EBV-infected human cells [114] and can also stabilize G4s in the LTR promoter to silence HIV-1 viral transcription in HIV-1-infected cells [115]. Meanwhile, many hnRNPs appear in

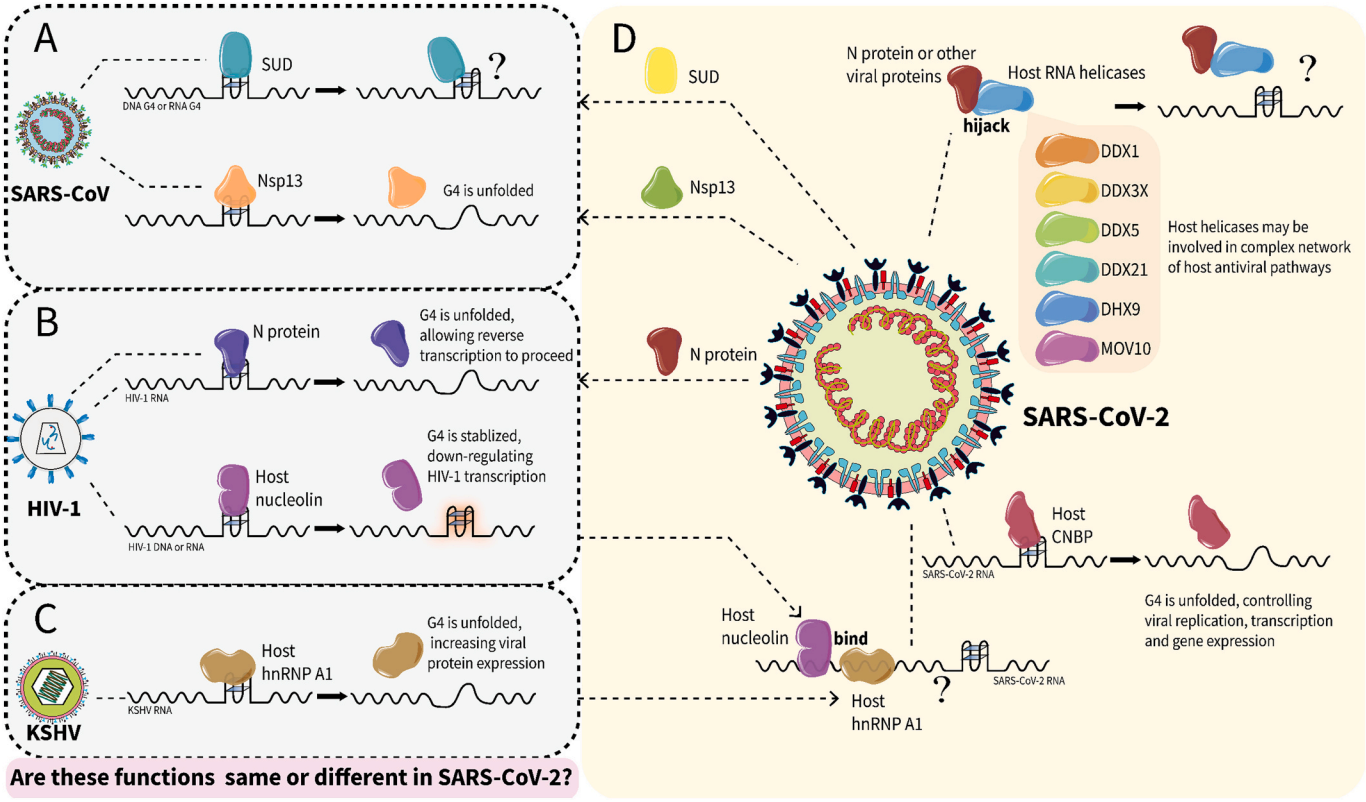


**Fig. 7.** The speculative interactions between SARS-CoV-2 G4s and proteins based on previous studies of other viruses. (A) In SARS-CoV, SUD binds G4; Nsp13 binds and unfolds RNA G4. (B) In HIV-1, N protein binds and unfolds RNA G4; host nucleolin binds and stabilizes G4. (C) In KSHV, host hnRNP A1 binds and unfolds RNA G4. (D) In SARS-CoV-2, viral N protein hijack host helicases; host CNBP binds and unfolds RNA G4; both nucleolin and hnRNP A1 bind to SARS-CoV-2 RNA. It is unknown whether these proteins in SARS-CoV-2 may have similar or different functions compared to other viruses.

the host interactome of SARS-CoV-2 RNA, including A0/A1/A2B1/A3/AB/D/H3/L/M [110,112,113]. In HIV-1-infected cells, hnRNP A2B1 unfolds LTR promoter G4s, thereby enhancing transcription of the HIV-1 genome [116]. In KSHV-infected cells, G4 in LANA1 mRNA inhibits its translation, while host hnRNP A1 increases LANA1 expression by selectively binding and unfolding G4s [117]. These studies suggest that nucleolin and hnRNPs may also have a similar effect on SARS-CoV-2. In addition, the interactome of SARS-CoV-2 RNA contains many host helicases, including DDX5/6/21/23/38B/42/46, DHX36/39B, MOV10, UPF1, and the previously discussed DDX3X, DDX21, DHX9, which can be hijacked by the N protein [110,112,113]. These RNA helicases may play an important role in regulating viral RNA G4s [118,119]. Finally, it is worth noting that in the study of Schmidt et al., CNBP was shown to be the most enriched SARS-CoV-2 RNA-binding protein, even surpassing N, S, M, and all Nsp proteins [112]. CNBP has also been reported to bind and unfold G4 formed in the SARS-CoV-2 genome *in vitro* [39]. Thus,

**Table 4**
Potential G4-interacting proteins in the interactome of SARS-CoV-2 genome.

| Origin | | Protein | Bind G4s | Unfold G4s | Stabilize G4s | Bind SARS-CoV-2 genome | Inhibitor | Reference |
|---|---|---|---|---|---|---|---|---|
| Virus | SARS-CoV | SUD | ✓ | | | | | [52,53] |
| | SARS-CoV-2 | SUD | ✓ | | | | | [55] |
| | | Nsp13 | ✓ | ✓ | | | ✓ | [33,75–77] |
| | | N | | | | ✓ | ✓ | [112,120] |
| Host | hnRNPs | hnRNP A1 | ✓ | ✓ | | ✓ | | [112,113,117] |
| | | hnRNP A2 | ✓ | ✓ | | ✓ | | [113,121] |
| | | hnRNP AB | ✓ | ✓ | | ✓ | | [112,113,122] |
| | | hnRNP A2B1 | ✓ | ✓ | | ✓ | | [111–113,116] |
| | | hnRNP D | ✓ | ✓ | | ✓ | | [113,123,124] |
| | | hnRNP F | ✓ | ✓ | | ✓ | | [113,125,126] |
| | | hnRNP H | ✓ | ✓ | | ✓ | | [113,126] |
| | | hnRNP P2 | ✓ | | | ✓ | | [113,127] |
| | RNA Helicases | DDX1 | ✓ | ✓ | | ✓ | | [112,128] |
| | | DDX3X | ✓ | | | ✓ | ✓ | [103,111–113,129,130] |
| | | DDX5 | ✓ | ✓ | | ✓ | | [113,131] |
| | | DDX21 | ✓ | ✓ | | | | [104] |
| | | DDX42 | ✓ | | | | | [132] |
| | | DHX9 | ✓ | ✓ | | ✓ | | [105,113] |
| | | DHX36 | ✓ | ✓ | | | | [105,133] |
| | | MOV10 | ✓ | ✓ | | ✓ | | [112,134] |
| | Other proteins | CNBP | ✓ | ✓ | ✓ | ✓ | | [112,113,135–138] |
| | | Nucleolin | ✓ | | ✓ | ✓ | ✓ | [113,139–147] |

CNBP appears to be crucial for SARS-CoV-2, and the available evidence seems to point to its unfolding activity on SARS-CoV-2 G4s, just as the helicase does. Altogether, we found these potential G4 regulators in the RNA interactome of SARS-CoV-2, and further studies are needed to uncover their detailed functions.

Here, we summarized the potential G4-binding proteins from both the virus and the host in Table 4. Now, this is a rapidly developing research field. When we were almost finished with this review, Mukherjee et al. reported modulation of the conformational space of RG-1 G4 in SARS-CoV-2 by cellular components and two amyloidogenic peptides, α-Syn and hIAPP, which are involved in Parkinson's disease and type-II diabetes [148]. These peptides stabilized RG-1 folding in a sequence-specific way, which was different from their interaction with human telomeric G4. The specific interaction may alter the expression profiles of disease-modifying genes and contribute to the development of specific ligands for intervention. Therefore, we look forward to the discovery of more specific interactions between SARS-CoV-2 G4 and proteins, which could be studied in-depth and applied to the treatment of SARS-CoV-2 in the future.

## 5. G4s as biosensors for SARS-CoV-2 detection

In addition to targeting SARS-CoV-2 G4s for antiviral therapy, we further discuss the possibility of using G4s for viral detection. Due to their high sensitivity, high stability, convenient operation, and low cost, G4 structures have been applied as novel biosensors to detect the surrounding conditions, including metal ions, pH, molecular crowding, *etc.* based on their conformational changes. The signal can be visualized by G4-specific dye molecules; alternatively, G4s combine with hemin to form the active DNAzyme, which then catalyzes the reaction between $H_2O_2$ and ABTS, leading to a color change for visualization [149]. It is worth noting that G4 structures have also been employed in the detection of pathogen genes as signal elements (Fig. 8). For example, Guo et al. reported a label-free biosensor for HIV-1 detection based on ligand-responsive G4 formation [150]. The target DNA formed a hybrid with the beacon, and then the released strand folded into G4 and could be visualized by NMM (Fig. 8A). Target DNA detection in HBV using PCR amplification was also established based on the sensitive G4 probe [151]. In addition, the ultrasensitive detection of HIV and HCV with G4-specific fluorescent or colorimetric probes as signal carriers have been realized using a DNA cascaded multiple amplification strategy [152].

In addition to their application in gene detection, G4-based aptamers have been developed against protein targets as recognition elements for diagnostic purposes (Fig. 8B-C). As a proof of concept, the thrombin binding aptamer (TBA) is the most well-studied G4-aptamer in protein detection due to its high affinity and specificity to the target. The thrombin binding triggers the rapid switching in the aptamer structure and induces the folding of an antiparallel G4 structure that can be detected by a variety of means (Fig. 8B) [153]. Using the first phase II clinical trial aptamer, AS1411, which can specifically bind nucleolin on the surface of cancer cells, an electrochemical sensor that can achieve label-free cancer cell detection was established [154]. The G4-aptamer was also applied to detect *Staphylococcus aureus* by targeting the presenting protein A on the cell surface, expanding the application of G4-aptamers as an analytical tool for the specific recognition and rapid detection of pathogens [155].

Considering the existence of PQSs in the SARS-CoV-2 genome, a G4-based biosensor has been proposed for SARS-CoV-2 detection in a recent review [156]. With the aid of molecular beacons comprising G4 sequences that can be folded after combining with the target gene, the SARS-CoV-2 gene may be detected as described in the above examples. In addition, identifying and utilizing the conserved PQSs in the SARS-CoV-2 genome may allow simple and direct virus detection (Table 5). For example, Josué Carvalho et al. designed two molecular beacons based on conserved SARS-CoV-2 G4s at positions 1574 and 24,268 to develop a faster, more sensitive, and inexpensive SARS-CoV-2 infection

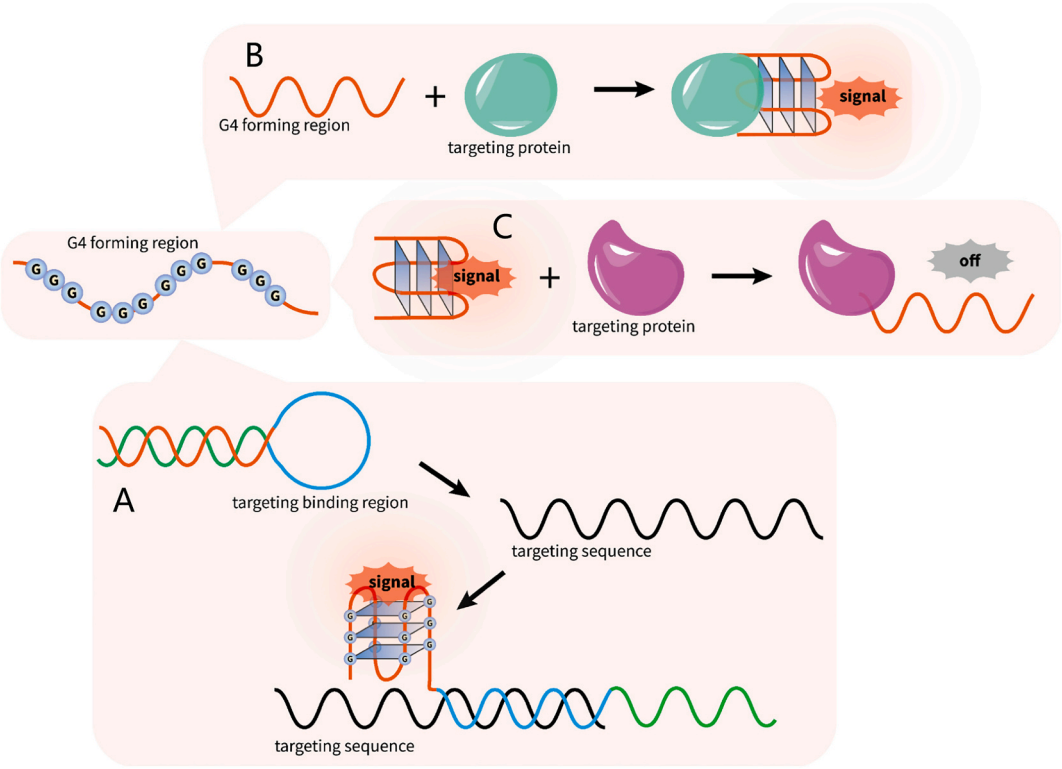


**Fig. 8.** The G4-based biosensor is a potential tool to detect the target gene and target protein. (A) The target gene formed a hybrid with the beacon, and then the released strand folded into G4 and could be visualized either by the fluorescent ligand or by the colorimetric method. (B—C) The binding of the target protein triggers the rapid switching in the G4-aptamer structure and induces the folding or unfolding of G4.

**Table 5**
The examples of G4s used in SARS-CoV-2 diagnosis [37,157,158].

| G4 origin | G4 sequence | Principle | Effect | The possible limitation to users |
|---|---|---|---|---|
| Two molecular beacons were designed based on the complementary sequences from SARS-CoV-2 RNA to detect G4s within the SARS-CoV-2 genome from human samples. | **1.GGTGTTGTTGGAGAAGGTTCCGAAGG**<br>**2.GGCTTATAGGTTTAATGGTATTGG** | Detect the G4 regions in ORF1ab and S genes in SARS-CoV-2 RNA by the molecular beacons based on their conformational rearrangements. | A fast (total duration of 2 h 20 min including amplification and fluorescence reading stages) and simple way of detecting SARS-CoV-2 in clinical samples. | Although the sensitivity (96.3 %) was similar to the current methods, the specificity (46.9 %) was still lower than the reference RT-PCR. |
| A conserved G4 DNA was obtained after reverse transcription and amplification from genomic SARS-CoV-2 RNA. | **GGTATGTGGAAAGGTTATGG** | Detect target G4 DNA by a fluorogenic probe named BTMA within an exclusive pH window (3.5–4.0). | A reliable strategy for a fluorogenic organic molecule-based platform to diagnose COVID-19 clinical samples. | A specific and noncommercial fluorescent probe is required, which was developed by the authors. |
| A 44-mer G4 aptamer against spike trimer antigen of SARS-CoV-2. | **TGGGAGCCTGGGACATAGTGGGGAAAGAGGGGAAGAGTGGGTCT** | The G4 aptamer binds tightly and selectively to the spike antigen of SARS-CoV-2. | 91 % sensitivity and 98 % specificity between SARS-CoV-2 infected individuals from the non-infected individuals. | Despite the low cost (~ $2–3 USD/test), the aptamer-based assay delivers sample-to-answer within 5 h. |

detection assay [37]. Recently, a highly conserved antiparallel DNA G4 sequence was identified from the SARS-CoV-2 genome, with unique loop compositions forming a distinct recognition motif. This sequence changes from the duplex form to the G4 structure in a pH-dependent manner. Thereafter, the authors developed a reliable fluorometric detection of SARS-CoV-2 by targeting this DNA G4 through pH-triggered conformational changes [157]. Furthermore, G4-based aptamers against proteins have been used in disease diagnosis and therapy [153]. As there are specific interactions between G4s and SARS-CoV-2-encoded proteins, such as Nsp13 and the SUD, G4-based aptamers may also be applied in SARS-CoV-2 detection. Recently, a 44-nt G4-forming aptamer was identified against the spike protein in SARS-CoV-2 [158], further reflecting the possibility of utilizing a G4-aptamer for SARS-CoV-2 detection (Table 5).

## 6. Conclusion and perspective

The G4 structure, as a promising drug target, has been widely applied in studies of disease treatment, such as cancer [159], parasite infections [160], neurodegenerative disorders [161], and virus infections [25]. In this review, we focus on G4s in SARS-CoV-2. Over the last two years, studies on the genomic PQSs of SARS-CoV-2 have emerged rapidly, reflecting that the G4 structure has received great attention in virus-related fields. We summarize all the reported SARS-CoV-2 PQSs to date in Tables 1 and 2. These PQSs still require in-depth structural and functional characterization.

In addition to the G4 structure itself, we also comprehensively introduce the G4-interacting proteins of SARS-CoV-2 from both the virus and the host cell, as summarized in Table 4. The SARS-CoV-2 SUD (Nsp3), Nsp13, and N proteins are the most significant virus-encoded G4 regulators. In addition, the host proteins that have been confirmed to be members of the SARS-CoV-2 RNA interactome and previously shown to act as G4 regulators in other viruses, such as helicases, hnRNPs, nucleolin, and CNBP, are also presented because these proteins may serve as potential drug targets to interfere with the normal functions of viral G4s. As the structures of some G4-binding proteins are already known, it is feasible to design inhibitors against these proteins to inhibit their interactions with G4s. Finally, we introduce the application of G4s as a possible approach to detect SARS-CoV-2.

It is worth noting that research in this area has just begun, and many questions are waiting to be resolved, including the following:

1) There are many predictions about the PQSs in SARS-CoV-2, but only some of them have been experimentally verified *in vitro*. The 3D structures of these G4s as well as their folding dynamics remain to be solved. Furthermore, whether all these PQSs can fold *in vivo* is also unclear.
2) RNA G4s may act as *cis*-acting elements in the process of SARS-CoV-2 transcription, replication, translation, and genome packaging, as shown in Fig. 2. However, the exact functions of RNA G4s in SARS-CoV-2 need to be further explored experimentally.
3) *Trans*-acting factors are required to resolve the RNA G4s in the viral life cycle, which are likely to be helicases. However, it is unknown whether host helicases or viral Nsp13 perform this function or whether there are more complex regulatory interactions between them. Whether host helicases are directly hijacked by viral RNA G4s or recruited through the N protein to viral RNA G4s is also not clear.
4) The identification and functional analysis of SARS-CoV-2 RNA G4-interacting proteins *in vivo* is needed. In particular, for host proteins, it is important to determine whether they are proviral or antiviral.
5) Using PDP to stabilize SARS-CoV-2 RG-1 and reduce N protein levels provided some clues regarding mechanisms of interference with viral G4s [38]. Whether the ligands can be effective in virus treatment still needs further verification. Moreover, as both viruses and hosts contain a large number of PQSs, designing ligands specific for virus G4s without affecting the normal function of cells is particularly important. This requires an in-depth study of the structures and folding mechanism of SARS-CoV-2 G4s.
6) In addition to SARS-CoV-2 RNA G4s, targeting G4-related proteins by inhibitors or G4-aptamers is also a plausible strategy for antiviral therapy.

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## Declaration of competing interest

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

## Data availability

Data will be made available on request.

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