Can G-quadruplex become a promising target in HBV therapy?

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The chronic infection with hepatitis B virus (HBV) is an important health problem that affects millions of people worldwide. Current therapies for HBV always suffer from a poor response rate, common side effects, and the need for lifelong treatment. Novel therapeutic targets are expected. Interestingly, non-canonical structures of nucleic acids play crucial roles in the regulation of gene expression. Especially the formation of G-quadruplexes (G4s) in G-rich strands has been demonstrated to affect many bioprocesses including replication, transcription, and translation, showing great potential as targets in anticancer and antiviral therapies. In this review, we summarize recent antiviral studies about G4s and discuss the potential roles of G4 structures in antiviral therapy for HBV.

Introduction

Chronic hepatitis B virus (HBV) infection remains a global health problem that impacts more than 250 million people (1). HBV infection can further progress to complications such as cirrhosis and hepatocellular carcinoma (HCC), which cause more than 780,000 deaths annually (2). Effective treatment of HBV is urgently needed to significantly reduce the progression of liver disease. Currently, there are two kinds of drugs for the treatment of HBV infection: interferons (IFNs) and nucleoside/nucleotide analogs (NAs). IFNs are a group of cytokines released by host cells to protect them from viral infection (3, 4), and they modulate the immune response against viruses by stimulating the expression of IFN-stimulated genes (ISGs). IFN therapy can inhibit viral replication, degrade viral components, and induce T-cell growth (5). However, only one third of the patients achieve a sustained response, and the undesirable side effects of IFN therapy are common, including influenza-like illness, fatigue, weight loss, anorexia, and emotional lability (2, 6). On the other hand, NAs directly target the reverse

transcription of pregenomic mRNA to DNA and inhibit the replication of HBV. But the change in envelope protein might induce immune escape (7), and the patients must face drug resistance during the indefinite treatment. Though many studies of the combination therapy of IFNs and NAs were conducted in recent years, there is still a lack of evidence on the effectiveness (8–11). A novel target for HBV therapy is required to improve current treatment.

Non-canonical structures of nucleic acids are now becoming promising targets in the research of various diseases. The secondary structures of nucleic acids are important for their function. Unlike double-helix DNAs, which are used to store genetic information, non-canonical structures, such as triplex, G4, and i-motif, are involved in the regulation of many bioprocesses (12). G4 is a typical non-canonical structure composed of π–π stacked G-tetrads, in which four guanines interact with each other through Hoogsteen base pairing (Figure 1A). G4-forming sequences are widely found in the human genome (13). Researchers have observed the presence of both DNA and RNA G4s in cells utilizing fluorescent G4-specific antibodies, and they could be targeted through G4 binding ligands (14, 15). G4s have various topological structures, which can be classified into parallel, antiparallel, and hybrid structures by the orientation of their strands (Figure 1B). Moreover, non-canonical G4 structures such as G4s with long loops or bulges are also commonly present, which might provide more binding sites for nucleotides, proteins, and ligands (13). The topology and stability of G4 are determined by not only its sequence but also the surrounding environment (16). For example, the presence of cations such as K^+ could significantly stabilize G4s (17). Under the molecular crowding conditions, the excluded volume effects of cosolutes, water activity, and the dielectric property of the solution are also crucial for G4s (18). The stability changes lead to topology changes or the folding or unwinding of G4s, thus affecting the

interaction between G4s and proteins and ultimately realizing their regulatory roles in gene expression. Correspondingly, in the analysis of the human genome, a great number of G4s are in regulatory regions such as promoters and untranslated regions, participating in multiple biological processes, including replication, transcription, and translation (19–22). Figure 2 presents the typical examples of G4s regulating gene expression, including the alteration of the interaction between promoter and functional proteins by G4s in the promoter region, the inhibition of transcription by G4s in the coding region, and the inhibition of translation by G4s in mRNAs (12).

Based on these characteristics, G4s have shown great potential in the research of many diseases. For example, G4s exist in many cancer-associated genes like Myc (23). They are important targets in anticancer studies due to their multiple functions, including the inhibition of oncogene expression, the suppression of telomerase activity, the selectivity for targeting cancer cells, and so on (24, 25). G4s have also been demonstrated to play key roles in viral infection, altering viral replication, transcription, and translation (26). On the other hand, G4-binding ligands attract great interest owing to their special binding affinity. The interaction between G4 and its ligand could significantly enhance or reduce the effect of G4 on biological processes, indicating the potential of G4s as a novel therapeutic target (27). In the past decades, a variety of G4 ligands have been developed through artificial synthesis or natural extracts. Li's group has built a database for G4 ligands since 2013 (28), and more than 3,000 kinds of G4 ligands were included with predicted ligand binding affinity and real-time ligand–receptor docking, providing a comprehensive tool for the discovery of drug candidates. Some G4-binding ligands such as CX-5461 have achieved significant development in clinical trials for safety, tolerability, and pharmacokinetics (29), further explaining the potential applications of G4s in future therapies.
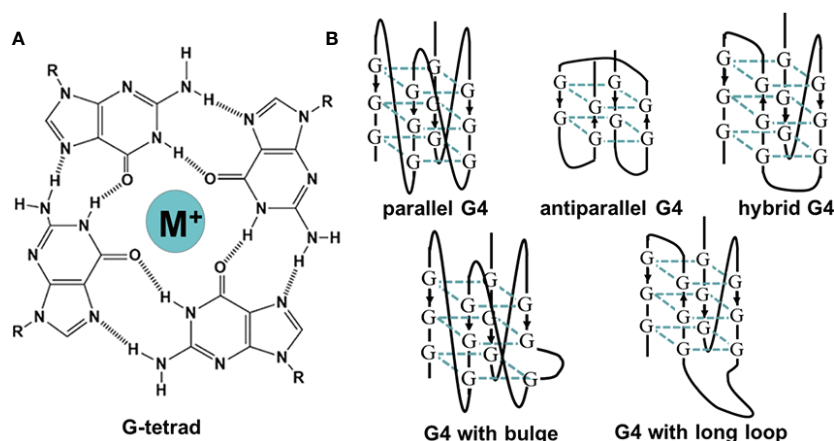

FIGURE 1
(A) The illustration of G-tetrad. (B) The schematic of G-quadruplexes with different topologies.
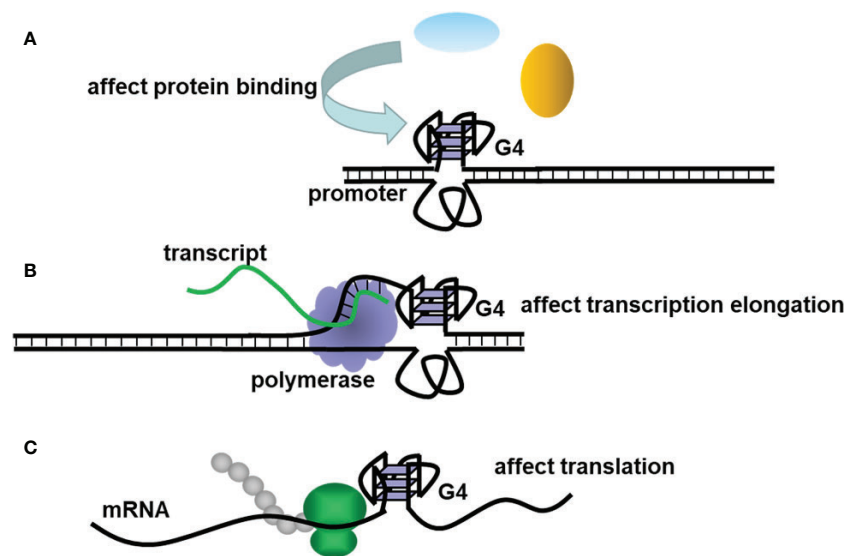
FIGURE 2
The schematic diagram of G4s regulating **(A)** promoter activity, **(B)** transcription, and **(C)** translation.

The antiviral effects of G4s make them promising targets for HBV-related studies (26). They might directly regulate viral gene expression, and the different therapeutic mechanism from IFN suggest the possibility of combination treatment. In this review, recent antiviral research on G4s was summarized, and the alternation of HBV gene expression was introduced in detail. The potential roles of G4s in HBV infection and treatment were discussed to provide a new perspective.

G4s as antiviral targets

G4s are promising antiviral targets due to their widespread existence in a variety of viruses and their regulatory function in gene expression. The study of G4s in viruses has started for decades since an intermolecular G4 structure was found in human immunodeficiency virus (HIV) genomic RNA in 1993 (30). Up to now, G4s have been widely found in the genomes of various viruses, including HIV, herpes simplex virus (HSV), hepatitis C virus (HCV), human papillomavirus (HPV), Zika virus (ZIKV), Epstein–Barr virus (EBV), Kaposi's sarcoma-associated herpesvirus (KSHV), severe acute respiratory syndrome coronavirus (SARS-CoV), human cytomegalovirus (HCMV), and so on (31). Table 1 lists examples of G4-forming sequences found in different viruses and their secondary structures, and the majority of G4s display parallel structures. Moreover, Lavezzo et al. searched for potential G4-forming sequences in the genomes of all known human viruses with a computational method (57). The potential G4 sequences were searched in patterns of $(G_2N_{1-7})_3G_2$, $(G_3N_{1-12})_3G_3$ and $(G_4N_{1-12})_3G_4$, and the results suggested that G4s were widely existing, and were more than expected from the composition. Double-stranded RNA and DNA viruses had a relative lack of G-rich islands compared with single-stranded viruses. It provided us with a comprehensive perspective on G4 presence in the viral study. With the development of genomics and bioinformatics, the existence of more G4s has been confirmed, and their biological functions have attracted the attention of researchers. The locations and functions of G4s in viral genomes are summarized in Table 2, and the interactions between G4 and G4 ligands/proteins were also involved.

G4s exist in both the noncoding and coding regions of viruses. Their functions are dependent on their locations. Promoter is one of the most common locations of G4s, suggested the importance of G4s in gene regulation. For example, researchers found G4s in the HIV-1 long terminal repeat (LTR) promoter, and the G-rich region overlapped with NF-κB and SP1 binding sites, showing crucial effects on promoter activity (34). Moreover, both DNA and RNA G4s were found in the U3 region of HIV-1, and multiple intermolecular G4s might form to affect the template switching of reverse transcription (37). G4s with long loops and bugles in the immediate early promoters of HSV 1 and 2 and varicella zoster virus (VZV) were identified to tune the promoter activity at the cellular level (75). In addition, G4s were found in other noncoding regions, such as the 3'-UTR in the ZIKV genome, which was essential for viral replication (40). G4s also widely existed in the coding regions of viral genes, which directly affected their transcription and replication. For example, there are reports describing the presence of G4s in nef, tat, rev, env,

TABLE 1 Examples of G4 forming sequences with structural characterization in different viral genomes.

Virus	DNA/RNA	G4 forming sequences (5'-3')	G4 structures	References
HIV-1	DNA	CTGGGCGGGACTGGGGAGTGGT	Parallel G4 with a T bulge	(32)
	DNA	TGGCCTGGGCGGGACTGGG	Antiparallel G4	(33)
	DNA	GGGGACTTTCCAGGGAGGCGTGGCCTGGGCGGG	Parallel G4 with long loops	(34)
	DNA	GGGAGGCGTGGCCTGGGCGGGACTGGGG	Hybrid G4 with a stem-loop	(35)
	DNA	GGAGGAGGAGGTGGG	Parallel G4	(36)
	DNA	GGGGGGACTGGAAGGG	Parallel G4	(36)
	RNA	AGG GAG GCG UGG CCU GGG CGG GAC UGG GGA GUG GC	Parallel G4	(37)
HCV	RNA	GGGCUGCGGGUGGGCGGGA	Parallel G4	(38, 39)
	RNA	GGGCAUGGGGUGGGCAGGA	Parallel G4	(38)
	RNA	GGGCGAAGGUCCUGGUGG	Parallel G4	(39)
	RNA	GGUGGAGGGUGAGG	Parallel G4	(39)
	RNA	GGGGGGGUGGGUGG	Parallel G4	(39)
	RNA	GGCGGAGGAGG	Parallel G4	(39)
	RNA	GGAGGUUAAGGCAGCGG	Parallel G4	(39)
ZIKV	RNA	GUGGAAGAGUGAUAGGACUCUAUGGCAAUGGGGUU	Parallel G4	(40)
	RNA	GUGGAGGUGGGACGGGAG	Parallel G4	(40)
	RNA	UCGGAUGUGGCAGAGGGGGCUGGAG	Parallel G4	(40)
	RNA	GCGGCGGCCGGUGUGGGGAA	Parallel G4	(40)
	RNA	GGAGUGGGAAGCGG AGCUGG	Parallel G4	(41)
	RNA	GGACCGCCUGGGGG UGGGGGGAGG	Parallel G4	(41)
	RNA	GGUGGGGGACUGG	Parallel G4	(41)
	RNA	GGCAUGGGGGAGG	Parallel G4	(41)
SARS-CoV-2	RNA	UGGCUGGCAAUGGCGGU	Parallel G4	(42)
	RNA	UGGAGGAGGUGUUGCAGGA	Parallel G4	(42)
	RNA	GGUAUGUGGAAAGGUUAUGG	Parallel G4	(43)
	RNA	GGCUUAUAGGUUUAAUGGUAUUGG	Parallel G4	(43)
	RNA	GGAUAUGGUUGGUUUGG	Parallel G4	(44)
HSV-1	DNA	GGGGCTGGGGCTGGGGTTGGGG	Antiparallel G4	(45, 46)
	DNA	GGGGGCGAGGGGCGGGAGGGGGCGAGGGG	Antiparallel G4	(45, 46)
	DNA	GGGAGGAGCGGGGGGAGGAGCGGG	Parallel G4	(45, 46)
	DNA	GGGGGAGAGGGGAGAGGGGGGGAGAGGGG	Parallel G4	(46)
EBV	DNA	GGGGGGGGTAGGGGGGGGG	Parallel G4	(47)
	RNA	AGGAGGUGGAGG	Hybrid G4	(48)
	RNA	AGGAGCAGGAGGUGG	Hybrid G4	(48)
	RNA	GGGGCAGGAGCAGGAGGA	Parallel G4	(49)
HCMV	DNA	GGGCCGGGACGGGGTGGG	Parallel G4	(50)
	DNA	GGGGTGGGAGGGACTTTTGCGGGTAGTGCATGCTAAGATGAACGGGTGGGCTGGGG	Parallel G4	(51)
	DNA	GGGGCACCCGGGTGTGGCGCTACGGG	Antiparallel G4	(51)
	DNA	GTGGTGTGGGGCCCGTGAGGGGGAGTCGTTGGG	Hybrid G4	(51)
KSHV	DNA	GGGGTGTGGGATGGGGGTGTGGG	Hybrid G4	(47)
	DNA	GGGTGGGAGGAGGAAGGATGTGGGGGTGGG	Parallel G4	(50)
	DNA	GGGGCGGGGGACGGGGGAGGG	Parallel G4	(52)
	DNA	GGGGCTCGGGGCTCGGGGCCCCGGGG	Parallel G4	(52)
HPV	DNA	GTGGGAGCGGGAACGGGAACGGGA	Parallel G4	(53)
	DNA	AGTGGTACAGGGGGTCGTACAGGGTACATTCCATTGGGTGGGCG	Parallel G4	(53)
	DNA	GGGAGTATGGGTAACGGGGGGGGG	Parallel G4	(53)

(Continued)

TABLE 1   Continued

Virus	DNA/RNA	G4 forming sequences (5'-3')	G4 structures	References
	DNA	GGGAAAGGGTACCTCGAGGGGCCGCGGGG	Parallel G4	(53)
	DNA	GGGTAGGGCAGGGGACACAGGGT	Hybrid G4	(53)
	DNA	GGGCAGGGTAGGGCAATTTAGGG	Hybrid G4	(53)
HBV	DNA	GGGAGTGGGGAGCATTCGGGCCAGGG	Hybrid G4	(54)
	DNA	CTGGGAGGAGCTGGGGGAGGAGA	Parallel G4	(55)
	DNA	GGCTGGGGCTTGGTCATGGGCCATCAG	Hybrid G4	(56)

and vpx coding regions in the HIV-1 gene (36, 87) and coding regions of prM, E, NS1, NS2, NS3, NS4B, and NS5 proteins in the ZIKV genome (40, 41). Many of them are important virulence factors, which are crucial for us to understand the effects of G4s in the viral life cycle and pathogenesis. Moreover, G4s in EBNA1 mRNA of EBV and LANA1 mRNA of KSHV were characterized to reveal the multiple functions of RNA G4s, such as the disruption of the interaction with the origin recognition complex and the control of translation to achieve immune recognition restriction (48, 49). The widespread existence and diverse function of G4 make it an important factor in viral gene expression.

Usually, G4 acts as a switch in the regulation of gene expression. The stabilization of G4s could significantly decrease the activity of the promoter and silence viral gene expression, while the unwinding of G4s could enhance the

TABLE 2   Overview of locations, functions of G4s in different viruses, and typical G4 binding ligands and proteins used in related studies.

Virus	Viral genome	Locations of G4s	Processes/Functions affected by G4s	Typical G4 binding ligands	Typical G4 interacting proteins	References
HIV-1	ssRNA (RT)	LTR promoter, 5' end of the gag gene, nef coding region	Promoter activity, transcription, nef protein expression, HIV-1 infectivity	BRACO-19, TMPyP4, c-exNDI	nucleolin, hnRNP A2/B1, HIV-1 nucleocapsid protein	(34, 36, 37, 58–62)
HCV	ssRNA (+)	HCV core gene, 3' end of negative strand	Replication, translation	PDP, TMPyP4, PDS, PhenDC3	Nucleolin, HCV nonstructural protein NS3	(38, 39, 63, 64)
ZIKV	ssRNA (+)	Coding regions for prM, E, NS1, NS2, NS3, NS4B, and NS5 proteins, 3'-UTR	Replication, viral protein synthesis	TMPyP4, BRACO-19, PDS		(40, 41, 65, 66)
SARS-CoV-2	ssRNA (+)	5' UTR, ORF1 ab, spike, ORF3a, membrane, and nucleocapsid genes	Viral life cycle, nonstructural proteins expression	TMPyP4, PhenDC3, BRACO-19, PDS, CX-3543, berberine	Nucleolin, CNBP, hnRNPs, RNA helicases, viral helicase nsp13	(42–44, 67–74)
HSV-1	dsDNA	Inverted repeats, immediate early promoter	Replication, transcription, viral production, recombination	BRACO-19, TMPyP4, c-exNDI		(45, 46, 75–78)
EBV	dsDNA	mRNA of EBNA1, BHRF1 promoter	EBNA1 mRNA translation, viral Bcl-2 expression	BRACO-19, PDS, PyDH2, PhenDH2, PhenDC3	Nucleolin	(47, 49, 79–81)
HCMV	dsDNA	Promoter regions including unique long, unique short, terminal and internal repeat regions, promoter of miR-US33	Viral gene expression, miRNA promoter activity	TMPyP4, NMM, CX-5461, PDS		(50, 51, 82)
KSHV	dsDNA	Terminal repeat region, LANA1 mRNA, promoter of KS-Bcl-2, promoters of miR-K12–1–9,11	Viral replication, LANA expression, viral Bcl-2 expression, miRNA promoter activity	PhenDC3, PhenDH2 TMPyP4, PDS		(47, 48, 50, 52, 83)
HPV	dsDNA	Long control region, coding region for L1, L2, E1, and E4	Viral protein production, viral gene expression	TMPyP4, BRACO-19, PDS, 360A, PhenDC3, C8		(53, 84–86)
HBV	dsDNA (RT)	PreS2/S promoter, coding region of polymerase protein, pre-core promoter of cccDNA	Promoter activity, protein production	TMPyP4, BRACO-19, PDS	DHX36	(54–56)

promoter activity and resume higher efficiency in viral transcription and replication (84, 88). Therefore, G4s are considered promising antiviral targets, and the study of G4 binding ligands has been one of the most attractive areas for years. Table 2 lists typical G4-binding ligands reported in recent antiviral research. BRACO19, TMPyP4, PhenDC3, and PDS are most used G4 ligands, which could significantly stabilize G4 formation and show antiviral activity. For example, the presence of BRACO19 was demonstrated to stabilize G4s in the LTR promoter, decrease its activity, and repress the transcription of HIV-1 (58). BRACO19 could also combine with G4s in the genome of EBV to suppress viral replication (89). G4 ligands and their biological effects in antiviral studies have been reviewed in detail, revealing the potential of G4 ligands in the development of novel antiviral therapies *via* G4-mediated pathways (27, 31, 90). However, some G4 ligands had relatively poor selectivity between G4s and duplexes or other quadruplexes, DNA G4s and RNA G4s, and different G4 structures, which hindered the clinical application of G4 ligands (27). Although there are still some problems with their application, G4 ligands do exhibit excellent antiviral activity. Further understanding of their mechanism would expand the pharmacological application of G4 ligands.

G4-binding proteins are also the focus of research because they are involved in replication, transcription, and mRNA processing (91). They could be divided into two groups, proteins stabilized G4s, and proteins unfolded G4s. Nucleolin is the most studied protein to stabilize G4s in antiviral research. For example, it was demonstrated to bind G4s in the LTR promoter and silence HIV-1 transcription (59). Nucleolin was also found to bind to HCV core RNA G4s and suppress HCV replication (39). On the other hand, proteins that can unwind G4s are essential for the replication and repair of viral genes. Viral-encoded proteins, such as HIV-1 nucleocapsid protein NCp7, HCV nonstructural protein NS3, and SARS-CoV nonstructural protein 13 (nsp13), were demonstrated to efficiently unfold G4s to allow effective replication and enhanced transcription (60, 63). Interestingly, the unfolding of G4s by these proteins could be reduced in the presence of G4 binding ligands such as PDS and BRACO-19, suggesting the potential of G4s and their ligands in antiviral therapies (60, 63). G4-binding proteins in host cells could also be utilized by viruses in their replication, which are considered better targets to achieve a wide response using common cellular pathways. For example, human nuclear ribonucleoprotein A2/B1 (hnRNP A2/B1) efficiently unfolds G4s to enhance HIV-1 transcription (61). Cellular nucleic acid binding protein (CNBP) was also demonstrated to interact with G4s in the SARS-CoV-2 RNA genome and promote their unfolding (44). These studies provide us with an advanced understanding of viral replication and transcription mechanisms and show the great potential of G4-targeted ligands and proteins in antiviral treatment.

In addition, making artificial G4s in the viral genome to inhibit viral gene expression is another application of G4s in antiviral research. For example, stable G4s have been demonstrated to block viral transcription and replication. A strategy using a tetraethylene glycol-modified oligonucleotide that could form an intermolecular G4 with the GG sequence in HIV-1 was designed to block the reverse transcription of HIV-1 and suggested a promising therapeutic method utilizing the function of G4 (92).

Roles of G4s in HBV genome

HBV is a partially double-stranded DNA virus with a relaxed circular DNA (rcDNA) genome of approximately 3.2 kb (93). As shown in Figure 3, in HBV infection, the virus interacts with its receptor, the bile acid transporter NTCP, and enters the cell. The rcDNA is then released into the nucleus and converted into covalently closed circular DNA (cccDNA). The transcribed pregenomic mRNA further translates to core proteins and polymerase and serves as the template for reverse transcription to rcDNA. The newly formed rcDNAs are enveloped and then released as new virions or recycled for cccDNA repair.

The confirmation of detailed G4 in HBV is relatively late compared with HIV. The first evidence of G4 formation in the HBV genome was reported in 2017. Biswas and coworkers analyzed full-length HBV sequences with the pattern $G_{3-4}N_{1-7}$ and found a G4-forming sequence in the presS2/S promoter of HBV genotype B (54). The structure was further confirmed as a hybrid intramolecular G4 by circular dichroism (CD), nuclear magnetic resonance, native polyacrylamide gel electrophoresis, and DMS footprinting analysis. The formation of G4 in the preS2/S promoter was related to promoter activity, and mutations in G4 resulted in the reduction of preS2/S transcripts and HBsAg levels. As HBsAg was the major surface protein in the HBV envelope, virion secretion was greatly affected by the presence of G4.

Meier-Stephenson and Badmalia discovered another unique G4 in the pre-core promoter of HBV cccDNA, which was the central part of pregenomic RNA generation and protein synthesis (55). A 23-mer oligomer in wild-type promoter region and three mutant types with a single-nucleotide mutation of G to A were characterized by CD and small-angle X-ray scattering. Parallel G4 structures were observed in both the wild and mutant types. Interestingly, the combination of G4 and the G4 binding protein DHX36 significantly pulled down HBV cccDNA. Moreover, the HepG2 transfection study demonstrated that, compared with the mutant ones, the expression of HBsAg and HBeAg was significantly decreased in the wild type. In contrast, the level of HBcAg was increased, suggesting the formation of G4 had different effects on viral core protein production.
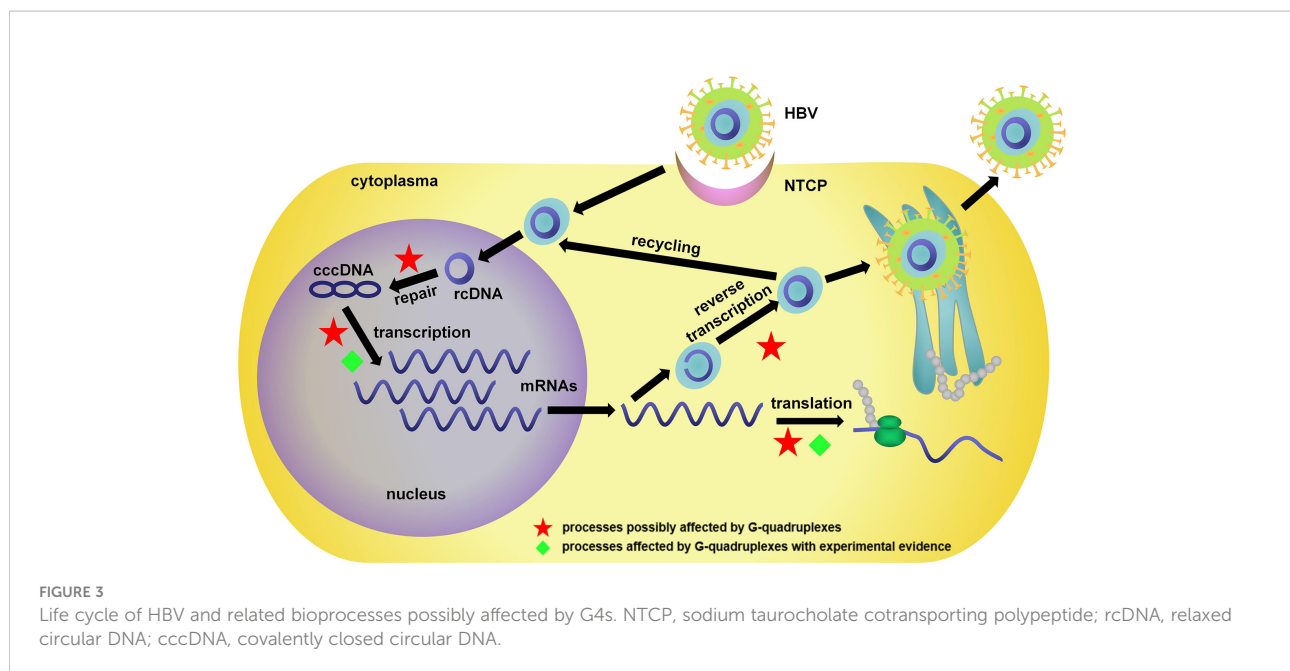
Molnár analyzed the effects of cations and G4 ligands on a G4 structure in the HBV genome (56). A hybrid G4 was observed in the presence of K^+, while a parallel structure was induced in the presence of Na^+, Li^+, and Rb^+. The interaction between G4 ligands TMPyP4, BRACO19, and PhenDC3 was characterized in detail as well. This provided more information about the possible roles of G4 and its ligands in the fight against HBV.
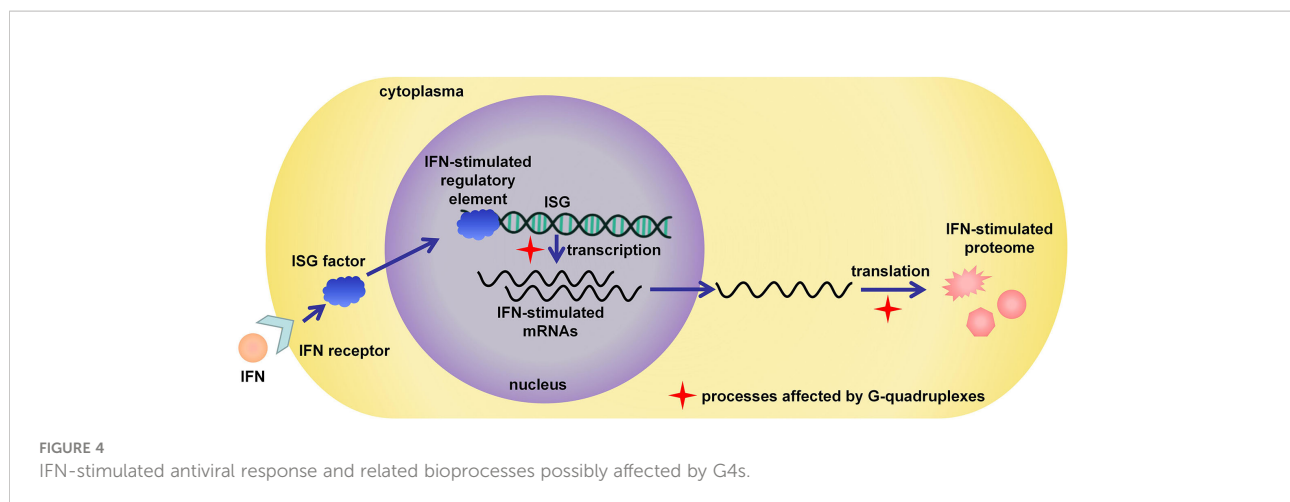
As a double-stranded DNA virus, HBV has a relatively low level of G4-forming sequences compared with single-stranded DNA/RNA viruses (57). Notably, the limited G4s in key regions could still be a crucial factor in the regulation of gene expression. The red stars in Figure 3 indicate processes possibly affected by G4s. These works provided sufficient evidence for the existence of G4s in the HBV genome, and as novel antiviral targets, their potential functions were investigated in HBV infection.

Roles of G4s in immune genes

Targeting G4s in related host genes is also a significant strategy in antiviral studies. Immune response is important in virus infection; therefore, ISGs are considered promising targets for antiviral therapy (94, 95). For example, IFNs released by host cells could stimulate the expression of ISGs to significantly suppress the infection and replication of the virus; therefore, IFNs have been approved for use in the clinical treatment of HBV infection (2, 6). Recent research has also revealed that the structure of nucleic acid is a critical factor involved in the immune response. For example, the long noncoding RNA lnc-MxA was reported to form a DNA/RNA triplex and inhibit the

transcription of IFN-β (96). G4, as an important functional non-B structure, showed great potential to affect the innate immune system and the expression of ISGs. The IFN-stimulated gene expression was shown schematically in Figure 4, and the red cross stars indicated the processes affected by G4s. The Seimiya group found that the formation of G4 in telomere repeats inhibited the expression of innate immune genes, including STAT1, ISG15, and OAS3 (97). Later, they reported that G4s bind to splicing factor 3B subunit 2 (SF3B2), suppress STAT1 expression and ISG induction (98). The induction occurred with various G4-forming sequences and could not be observed in the presence of non-G4 sequences, even though they had the same lengths or guanine contents as G4-forming sequences. Moreover, the presence of the G4 ligand Phen-DC3 blocked the interaction between SF3B2 and G4s and reversed the inhibition of ISG induction. It demonstrated that G4s were important for ISG expression and might be promising therapeutic targets in antiviral studies.

The effects of G4 on ISG were also investigated in HBV infection. Sun reported a novel mechanism of STAT1 involved IFN signaling based on RNA structural change (99). Based on the work of Kwok on RNA G4 sequencing in the human transcriptome (100), a G-rich sequence at the 5' UTR in STAT1 mRNA was found to form RNA G4 and participate in the regulation of STAT1 expression. Importantly, DDX5, an RNA helicase involved in transcription, RNA splicing, and translation, was demonstrated to selectively bind to the G4 structure in STAT1 mRNA and promote the translation of STAT1 protein. Notably, in the IFN treatment of HBV infection, the knockdown of DDX5 decreased the level of STAT1 protein because of G4 formation in mRNA, and

FIGURE 4
IFN-stimulated antiviral response and related bioprocesses possibly affected by G4s.

resulted in the reduction of IFN-α antiviral effect. This research discussed how the RNA structural changes affected IFN response, providing us a novel insight into the mechanism of IFN treatment.

Discussion

Non-canonical structures of DNAs and RNAs are promising targets in antiviral therapy, especially G4s. The presence of G4s has been demonstrated in various viral genomes to regulate their expression. In HBV studies, targeting G4s could regulate the development of HBV infection in two ways. One is targeting G4s located in the promoter regions of the HBV genome, which can regulate viral replication and transcription. Mutations that disrupt the formation of G4s could significantly decrease the expression level. G4-binding proteins and G4 ligands are promised to control the viral life cycle by blocking the interaction between G4 and host proteins in the initial stages of replication and transcription. The other one is targeting G4s in ISG genes. The presence of G4s could inhibit the transcription of ISGs, thus affecting the therapeutic effect of IFN treatment. The fact that the repression of ISGs could be reversed by G4-binding proteins and ligands suggested their potential in the modulation of immune response. With the development of more G4s in HBV and related ISGs, our understanding of their function in the viral life cycle and HBV treatment will improve.

In addition, G4s are involved in other functional RNAs, such as miRNAs and lncRNAs (101–103). Notably, both miRNAs and lncRNAs play key roles in HBV infection and HBV-related HCC, suggesting G4s and their ligands could affect the progress of HBV and HBV-related HCC through novel routes (104–107). For example, metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is a non-coding RNA that regulates alternative splicing in HBV-related HCC (104). Three parallel G4s were recently found in the 3' region of MALAT1, which

interacted with nucleolin and affected the alternative splicing of RNA transcripts (103). Similar results were also observed in miRNA studies. The formation of G4 in miRNA was demonstrated to affect miRNA-mRNA interaction and regulate miRNA-mediated post-transcription (108). The G4s in functional RNAs might provide a new perspective for us in the development of novel targets in HBV therapy.

However, there are still some challenges to overcome before targeting G4s in the clinical treatment of HBV infection. First, most of the thermodynamic analysis of G4s was conducted *in vitro*. As the stability of G4 is greatly affected by its surrounding environment, whether *in vitro* experiments accurately mimic the internal environment of infected cells is a problem. Notably, analytical methods have been built to achieve the visualization of G4s and their structural transition utilizing G4 antibodies or fluorescent probes in recent years (14, 109). More valuable information about the structural change and regulation mechanism of G4s in cells is expected in the future. Second, the selectivity of G4 ligands might be a potential risk factor. There has been evidence showing that some G4 ligands could bind to other non-canonical structures like the i-motif (110). It was suggested that the mechanism of G4 ligand-induced effects might be complicated. Third, it is difficult for G4 ligands to bind with a specific G4. This means they might target multiple G4s in different locations in the genome at the same time. In the comparison of the interactions between different G4s and typical ligands, including TMPyP4, BRACO19, PDS, PhenDC3, and TrisQ, none of them showed obvious selectivity for a specific topology (111). Now researchers are trying to improve the selectivity of G4 ligands to avoid unexpected side effects or cellular toxicity (112). Moreover, more clinical data is necessary for antiviral application. Now the clinical trials of G4 ligands are mainly about anticancer therapy, and some of them have achieved significant progress (29). G4s and their binding proteins/ligands could directly affect the expression of viral genes; therefore, as promising targets, G4s should not be

ignored in HBV therapy. Regardless, G4-related research provides a special perspective on HBV treatment, and novel strategies targeting the regulation of G4s are expected in the future.

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