A highly conserved G-rich consensus sequence in hepatitis C virus core gene represents a new anti–hepatitis C target

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G-quadruplex (G4) is one of the most important secondary structures in nucleic acids. Until recently, G4 RNAs have not been reported in any ribovirus, such as the hepatitis C virus. Our bioinformatics analysis reveals highly conserved guanine-rich consensus sequences within the core gene of hepatitis C despite the high genetic variability of this ribovirus; we further show using various methods that such consensus sequences can fold into unimolecular G4 RNA structures, both in vitro and under physiological conditions. Furthermore, we provide direct evidences that small molecules specifically targeting G4 can stabilize this structure to reduce RNA replication and inhibit protein translation of intracellular hepatitis C. Ultimately, the stabilization of G4 RNA in the genome of hepatitis C represents a promising new strategy for anti–hepatitis C drug development.

INTRODUCTION

Viruses rapidly mutate, and RNA viruses (particularly riboviruses, rather than retroviruses) have higher genetic variation than DNA viruses (1). Hepatitis C virus (HCV) infection is estimated to affect 2.8% of the population worldwide (2). Recent medical studies have focused on the development of small molecules targeting viral enzymes (3, 4). However, resistance rapidly emerges in HCV patients who are treated with specific inhibitors targeting nonstructural proteins, reflecting the error-prone replication by NS5B, a viral RNA-dependent RNA polymerase (RdRp) (5). Hence, the development of compounds targeting highly conserved regions or structural motifs in the HCV genome would have the most potential to protect against not only the current virus but also the varied ones (6, 7). Moreover, the use of small-molecule RNA binders as therapeutics is an area of intense interest at the interface of chemistry and biology (8, 9).

G-quadruplex (G4) contains stacked planar G-quartets, which are stabilized through Hoogsteen hydrogen bonding (fig. S1) (10–12). G4 RNAs have been identified in telomeric transcripts (13) and 5’ or 3’ untranslated regions (UTRs) of mRNAs (14–16). Recently, G4 RNAs have been associated with some viruses (17) such as HIV (18), herpes simplex virus (HSV) (19), and Epstein-Barr virus (EBV) (20). BRACO-19, a G4-binding ligand, has been demonstrated to be an active anti-HIV agent (21). However, for HSV and EBV, G4 RNAs only form in nascent RNA transcripts during transcription, and HIV only exists in the latent form of proviral DNA upon entry into human cells. To the best of our knowledge, the roles of G4 RNA in these studies are confined to the regulation of transient gene expression at the translational level. Until recently, there have been no reports concerning the presence of G4 structure in riboviruses. Because RNA replication and translation occur on the same RNA of positive-sense single-stranded RNA (ssRNA) viruses, we suggest that HCV G4 RNA (if present) play a more direct role in regulating both of these processes.

Here, we provide the first evidence that a highly conserved guanine-rich (G-rich) sequence is present in the HCV core (C) gene. Using various methods, we demonstrate the high potential of HCV C consensus sequences to form G4 RNAs. Furthermore, our biological findings provide direct evidences that, because of the actions of G4 ligands in binding to and stabilizing G4 RNA in the specific G-rich region of the C gene, they can reduce RNA replication and inhibit protein translation in HCV. Ultimately, the highly conserved nature of HCV G-rich RNA might represent a challenging new target for anti-HCV drug development.

RESULTS

Bioinformatics analysis to reveal HCV G-rich sequences

On the basis of a genomic variance analysis, HCV was classified into multiple genotypes (1 to 7) with varied subtypes (22). We retrieved 77 complete genomic sequences of all available genotypes (GenBank accession numbers in table S1) and conducted bioinformatics analysis to assess the level of sequence conservation (23). As shown in fig. S2, a low ratio (30.89%) of conserved nucleotide sites across the whole genome was observed, whereas much higher conservation (46.77%) was identified within the nucleotide sites of the C gene. Thus, we are particularly interested in the development of a new antiviral strategy targeting the HCV C gene.

We next performed multiple sequence alignments of the C gene across these 77 HCV genomes (figs. S3 to S5). In this example, a G-rich consensus sequence harboring four G-tracts was observed in the central region of the C gene, between positions +259 and +285. A high proportion (47.37%) of the nucleotide sites is conserved across such a G-rich region, which is highly characteristic of sequences with high potential to form G4 structures. HCV subtypes 1a and 1b are commonly observed in
East Asia and North America (24), prompting significant interest in the study of these specific subtypes within the field. Hence, we aligned 1056 partial coding sequences (cds) of the C gene for HCV subtype 1a and 1025 cds for HCV subtype 1b (tables S2 and S3), retrieved from the HCV database. WebLogo was used to generate a graphical representation of the aligned sequences (25) between positions +267 and +285. As illustrated in Fig. 1, RNA1a (GGGCUGCGGGUGGGCGGGA, 735/1056) and RNA1b (GGGCUGCGGGUGGGCGGGA, 656/1025) were among the sequences most frequently observed to display great potential for G4 formation; therefore, these sequences were selected as targets for the following studies.

G4 RNAs evidenced through gel electrophoresis and $^1$H nuclear magnetic resonance
Native polyacrylamide gel electrophoresis (PAGE) was performed using fluorescently labeled RNAs (RNA1a-FAM and RNA1b-FAM in Table 1) to monitor structure compaction, as G4 RNA should migrate faster than ssRNA (26). As shown in Fig. 2A, the band corresponding to target RNA migrated significantly faster than its G4-mutated counterpart, indicating the formation of a stable unimolecular structure. Compared with RNA1b-FAM, RNA1a-FAM migrated significantly faster, indicating a more compact structure for RNA1a. As there are only two Gs located in the fourth tract of RNA1b, the structure of this RNA is expected to be less stable than that of RNA1a, which contains three stacked G-quartets.

To further confirm the G4 structures, we conducted $^1$H nuclear magnetic resonance (NMR) analysis using chemically synthesized RNAs (RNA1a and RNA1b) (13, 27). Imino proton peaks within the 10.0- to 11.5-ppm region are highly characteristic of the Hoogsteen hydrogen bonds of G-quartets (28). Accordingly, the $^1$H NMR spectrum of RNA1a revealed well-resolved imino peaks within this region (red spectrum in Fig. 2B and fig. S6), indicating the formation of G4 structures. The $^1$H NMR spectrum of RNA1b demonstrated broad imino peaks within the same range (red spectrum in Fig. 2C and fig. S7), suggesting inhomogeneous G4 RNAs. Because there were only two G bases at the fourth G track, a two-G-tetrad motif was the most probable structure for RNA1b. Hence, RNA1b could potentially be involved in multiple alternative folding patterns (29). Furthermore, the effects of antisense oligonucleotides (ASOs), including AS-RNA1a and AS-RNA1b (Table 1), were examined. When RNA1a was probed with AS-RNA1a, additional peaks appeared at the lower field of 11.5 to 13.5 ppm (blue spectrum in Fig. 2B), suggesting the partial conversion of G4 into double-stranded RNAs (dsRNAs) (20). A similar phenomenon was also observed for RNA1b and AS-RNA1b (blue spectrum in Fig. 2C).

In a long RNA structural context, the folding of a specific structure might be influenced by the neighboring sequences (30, 31). Mfold Web server is used for folding analysis of the primary sequence of the HCV C gene (32). The results indicated that RNA1a and RNA1b are located in a very structured (dsRNA) region (blue zone in figs. S8 and S9). Here, longer RNAs (RNA1a Long and RNA1b Long in table S4) containing the sequence in the green area (positions +264 to +311; figs. S8 and S9) were synthesized. Although most of the peaks are centered in the “ds region,” the NMR results showed that the short motifs, including RNA1a and RNA1b, were capable of forming G4 structures (red spectrum in fig. S10). Moreover, G-A mutations largely disrupted the G4 formation (blue spectrum in fig. S10).

**Highly stable parallel G4 RNAs of the target HCV sequences**
To confirm the formation of G4 structures in the target HCV sequences, we performed circular dichroism (CD) analysis. As shown in fig. S11, the recorded spectra were consistent with parallel G4 structures (33). For RNA1a, the high stability of G4 RNA was confirmed through small variations of CD spectra observed at elevated temperatures (fig. S12A), whereas the spectra of RNA1b showed more variation within the same range (fig. S12B), indicating a less stable G4 RNA structure.

Next, we measured the spectra of these sequences in the presence of NaCl or LiCl. As shown in fig. S13, the spectra of the sequences were similar to those recorded in KCl solution, indicating parallel G4s.

Furthermore, CD spectroscopy of RNA1a revealed a minimum peak at 210 nm in the presence of AS-RNA1a (fig. S14A), which was highly reminiscent of the spectral signature associated with the A-form of dsRNA. Moreover, increasing the level of AS-RNA1a promoted a shift from 264 to 275 nm, indicating a structural switch alleviated from G4. When RNA1b was titrated with less AS-RNA1b, the minimum at 210 nm could be evidently observed (fig. S14B), confirming the increased G4 stability of RNA1a compared with RNA1b.

**Table 1. Sequences of some oligomers used in our studies.**

<table>
<thead>
<tr>
<th>Oligomer</th>
<th>Sequence (from 5’ to 3’)</th>
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<tbody>
<tr>
<td>RNA1a</td>
<td>5’-GGGCUGCGGGUGGGCGGGA-3’</td>
</tr>
<tr>
<td>RNA1b</td>
<td>5’-GGGCAUGGGUGGGCGGGA-3’</td>
</tr>
<tr>
<td>RNA1a-FAM</td>
<td>5’-FAM-AGGGCUGCGGGUGGGCGGGA-3’</td>
</tr>
<tr>
<td>RNA1a-Mut-F</td>
<td>5’-FAM-AGGGCUGCGGGUGGGCGGGA-3’</td>
</tr>
<tr>
<td>RNA1b-FAM</td>
<td>5’-FAM-AGGGCUGGGUGGGCGGGA-3’</td>
</tr>
<tr>
<td>RNA1b-Mut-F</td>
<td>5’-FAM-AGGGCUGGGUGGGCGGGA-3’</td>
</tr>
<tr>
<td>AS-RNA1a</td>
<td>5’-UCCCGCCCACCACCCGGACGCCC-3’</td>
</tr>
<tr>
<td>AS-RNA1b</td>
<td>5’-UCCUGGCCCAACCACCCGACGCCC-3’</td>
</tr>
</tbody>
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**Fig. 1.** Graphical representations of G-rich sequences consensus in the HCV genome. (A and B) A total of 1056 partial cds of the C gene for (A) subtype 1a and 1025 partial cds of the C gene for (B) subtype 1b were retrieved from the National Center for Biotechnology Information Web site (www.ncbi.nlm.nih.gov) and the HCV database (www.hcv.lanl.gov/) and aligned using WebLogo software.
Next, we examined whether ligand-mediated G4 stabilization can regulate RNA replication. In the HCV life cycle, the viral RNA is replicated by RdRp NssB (39). For the simulation of this process, an RNA stop assay is designed (Fig. 4A) using 3Dpol, which is an RdRp with a primer-dependent mechanism (42, 43). The HCV G-rich segments (red part in Fig. 4A) have been incorporated into the 5' ends of RNA templates, and the 5' FAM-labeled primer p15 (green part in Fig. 4A) is designed to target the 3' end of templates (blue part in Fig. 4A). Upon the addition of enzyme and nucleotide triphosphate (NTP), p15 is extended along the complementary template RNAs. When no G4 ligand is present, full extension can be achieved (left half in Fig. 4A). In contrast, if the binding of G4 ligands occurred, the extension would be stopped at the G4 site (right half in Fig. 4A).

As shown in lane 3 of Fig. 4B, fully extended products were observed (due to G4 formation) in the absence of G4 ligand. However, when increasing amounts of G4 ligand were incubated with template 1a-G4, the template-directed primer extension was gradually inhibited at the G4 site (lanes 4 to 7 in Fig. 4B). On the contrary, the site-specific termination event was not characterized for G4-mutated templates upon the addition of G4 ligand (lanes 10 and 11 in Fig. 4B). The G4 site-specific blockade in RNA synthesis was also detected when template 1b-G4 was treated with G4 ligands (fig. S24). Consistent with the results of the melting studies, ligand PDP exhibited superior G4 stabilization and polymerization inhibition.

Inhibition of the full-length C gene expression through G4 RNA stabilization
To investigate the effects of G4 RNA stabilization on the expression of a full-length HCV C gene, we performed Western blot analysis. Plasmid 24480 (pMO29) carrying the genotype 1b C gene (fig. S25) and the conserved backbone [pcDNA3.1(+)/pEV 204-Hind III] was used (44). As demonstrated in Fig. 4C, both PDP and TMPyP4 evidently inhibited the expression level of the HCV C gene. To better understand the mechanism behind the inhibition of HCV C gene expression through G4 ligands, we examined TMPyP2 (structure in fig. S19), a positional isomer of TMPyP4 with low affinity for G4 DNA (38). As demonstrated in fig. S26, TMPyP2 showed much less G4 RNA-stabilizing activity toward RNA1b, contained in pMO29. Comparisons of TMPyP2 (lane 4 in Fig. 4C) and TMPyP4 (lane 1 in Fig. 4C) on the basis of HCV C gene expression were consistent with the above results. To demonstrate that the observed effects are specific for G4 sequence, we prepared a
relevant control plasmid with a G4-mutated sequence. The following results (fig. S27) indicate that both PDP and TMPyP4 did not inhibit the translation of the G4-mutated C gene. Together, these results suggest that because of the actions of G4 ligand in binding to and stabilizing G4 RNA in the indicated region of the C gene (fig. S25), it is a promising candidate for further study.

Repression of enhanced green fluorescent protein expression through G4 RNA stabilization

Next, an enhanced green fluorescent protein (EGFP) reporter gene system was built to examine whether ligand-mediated G4 stabilization can regulate protein expression. We constructed a variety of pEGFP-C1 derivatives (45) after cloning the 21-bp sequences, containing either wild-type HCV G-rich sequences or the G-to-A mutant sequences. Plasmids GFP-1a core G4, GFP-1b core G4, GFP-1a core Mut, and GFP-1b core Mut were prepared, and the insert was placed immediately downstream of the human cytomegalovirus immediate early promoter (1 to 589) and upstream of the EGFP-cds present on the parental plasmid (figs. S28A and S29A). We next performed a confocal fluorescence assay. Upon addition of G4 ligand, the expression of EGFP in GFP-1a core G4 or GFP-1b core G4 was significantly inhibited compared with the DMSO treatment (fig. S28B), whereas the expression of EGFP in G4-mutated plasmids or the empty vector was not influenced under the same conditions (fig. S29B). Consistent with the previous results, TMPyP2 did not inhibit the expression of EGFP (fig. S28B). Together, the results suggest that G4 ligands inhibit reporter gene expression by targeting HCV G-rich RNAs.

To quantitatively evaluate the effects of G4 ligands on EGFP expression, we performed flow cytometry, and the results showed that EGFP was expressed much less efficiently in GFP-1a core G4– or GFP-1b core G4–transfected cells following G4 ligand treatment compared with the DMSO control (fig. S28C). In contrast, G4 ligands had a much less pronounced effect on EGFP expression in G4-mutated plasmids. Consistent with previous results, PDP showed better inhibition of EGFP expression.

**G4 ligands inhibit HCV replication in cells**

Next, we investigated whether G4 ligands can inhibit HCV infection. Currently, the most commonly used infectious HCV culture system is based on JFH1 (Japanese fulminant hepatitis 1, genotype 2a) (46),...
which undergoes efficient replication in Huh-7 cells and other cell lines (47). We analyzed the sequence of the C gene in the JFH1 genome and also identified the G4-forming sequence around the same region (fig. S30). A similar sequence alignment of the C gene for subtype 2a was further performed (table S6 and figs. S31 to S33), and a conserved G4 motif was also identified (fig. S34). The 1H NMR spectrum of RNA2a demonstrated broad imino peaks within the 10.0- to 11.5-ppm range (red spectrum in fig. S35A), providing direct evidence for G4 formation.

G4 mutations in the HCV C gene disrupt G4 ligand–virus interactions

To further demonstrate the existence of G4 RNAs in the HCV genome under physiological conditions, we used a pull-down strategy (37, 55). Because the introduction of a biotin affinity tag on PDP has been successfully applied in the selective enrichment of G4 DNAs (37), the same molecule (structure in fig. S42) was used in this assay. As demonstrated in fig. S43, biotin modification on PDP did not impair the stabilization of HCV G4 RNAs. Moreover, a pJ6/JFH1–G4-Mut plasmid containing a G4-mutated sequence in the C gene was prepared using overlapping extension PCR (OE-PCR). The transcribed J6/JFH1 or J6/JFH1–G4-Mut RNA was incubated in the presence or absence of biotin-PDP and sonicated to shear the genomic RNA. Subsequently, hydrophilic streptavidin-coated magnetic beads were used to capture the desired G4 RNAs (scheme in fig. S44). As shown in fig. S45, an evident peak at approximately 264 nm, representing parallel G4 RNAs, was observed for recovered wild-type viral RNA (green line), whereas almost no signals were observed for G4-mutated viral RNA (blue line) and the control sample without using biotin-PDP (black line).

Next, we performed RT-qPCR to determine the abundance of the HCV G4 motif in RNA samples before and after pull-down manipulation (57), using G4-fwd and G4-rev (sequences in table S4). The normalized results revealed an approximately 28-fold enrichment (ΔΔCt = 4.82), whereas the control assay that did not use biotin-PDP did not display any enrichment.

Next, we separately delivered the transcribed J6/JFH1 and J6/JFH1–G4-Mut RNA into Huh-7.5.1 cells through electroporation. The cells were infected and treated with various G4 ligands, and the replicated viral RNA levels were determined relative to the host cell GAPDH mRNA. As demonstrated in Fig. 6A, much more attenuated infections were observed for the J6/JFH1–G4-Mut virus. Western blot analysis further indicated that G4 ligands strongly inhibited Core protein expression of the wild-type, but not of the G4-mutated, J6/JFH1 (Fig. 6B). These results provided direct and solid evidence that G4 RNA in the HCV C gene represents a cellular target for typical G4 ligands such as PDP.

To further confirm whether the G4 motif is a molecular target of G4 ligands, we evaluated the activity of these molecules toward a different natural virus without a G4-forming sequence. Influenza A virus, a negative-sense ssRNA virus, was examined (58). As expected, G4 ligands were much less effective on this virus strain (fig. S46).

Previous investigations demonstrated that ASOs can bind to G4 RNA and affect specific mRNA translation (20, 59). Here, ASOs complementary to G4 were delivered into different HCV-infected cells. The results clearly demonstrated that such ASOs can destabilize the HCV G4s and show an opposite effect on RNA replication when using G4 ligands (fig. S47). As expected, the stimulatory effect on viral replication was significantly alleviated using mutant ASOs. The effect
observed for G4-mutated virus was nearly abolished in the presence of ASO targeting the same nucleotide position.

Full HCV genome forms G4 at the targeted site in cells
Because G4 structures have been visualized in human cells using antibody-based fluorescence imaging (11, 60, 61), we applied the same strategy to verify whether the target sequence truly formed G4 in cells. The well-developed G binding antibody BG4 (11) was used in these experiments. We first transfected FAM-labeled target RNAs into HuH-7.5.1 cells (fig. S48). After fixation and permeabilization, amplified red fluorescence indicating G4 formation was generated (the second column of images) according to a previously described method (60). Yellow regions indicating colocalization (indicated by white arrows in the rightmost column of images) were detected in the cytoplasm of cells.

**Fig. 5. G4 ligands suppress intracellular HCV replication.** (A) RT-qPCR was used to determine the amount of HCV RNA in HCV Con1/JFH1-infected HuH-7.5.1 cells treated in triplicate with the G4 ligands or control (DMSO or IFN-α). IFN-α was used at 150 ng/ml. The values observed were normalized to GAPDH. All data are presented as the means ± SEM from three independent experiments. The error bars reflect the SD. G4 ligand groups versus DMSO group, *P < 0.05. The primers were designed to target the C gene of Con1/JFH1 RNA. (B) RT-qPCR was performed, and the primers were designed to target the 5′UTR of Con1/JFH1 RNA. (C) Western blot analysis showed the suppression of intracellular HCV replication. A commercial anti–HCV Core 1b antibody was used, and the values indicate the percentage of densitometry of the target HCV protein relative to β-actin. (D) Western blot analysis was performed, and a commercial anti–HCV nonstructural protein 3 (NS3) antibody was used for detection.

**Fig. 6. G4-disruptive mutations in the HCV C gene inhibit G4 ligand–virus interactions.** (A) RT-qPCR was performed. The primers were designed to target the C gene of J6/JFH1 virus. All data are presented as the means ± SEM from three independent experiments. The error bars reflect the SD. (B) Western blot analysis was performed. The values indicate the percentage of densitometry of the target HCV NS3 protein relative to β-actin. Lane 1, no HCV control; lanes 2 to 7, J6/JFH1–G4-Mut virus; lanes 8 to 13, J6/JFH1 virus.
Moreover, the colocalization was inhibited after the ASO (targeting G4 site) treatment (the second, fourth, and sixth rows of images). We did not observe the colocalization (bottom row of images) using the G4-mutated sequence (RNA1b–Mut-F). The results provided strong evidence that BG4 binds to intracellular G4 structures formed in target RNAs.

To confirm that the target G4 motif was targeted by the G4 ligand in living cells, we performed further tricolor confocal microscopy (fig. S49). The signals for biotinylated G4 ligand (biotin-PDP) were developed using allopurinol (APC)-conjugated streptavidin (the midmost column of images). As shown in the merged channel (the rightmost column of images), the overlapping regions of red, green, and blue generated white images (indicated by white arrows), indicating the binding of biotin-PDP, BG4, and target G4 RNA in cells. In addition, the ASO (targeting G4 site) treatment inhibited the observed colocalization (the second, fourth, and sixth rows of images). Hence, target G4 RNAs were directly targeted by the G4 ligand.

To further verify whether the full HCV genome forms G4 at the target site in cells, we performed intracellular tracking of G4 in HCV (fig. S50). The ASO complementary to the adjacent sequence upstream of the target G4 site (probe1a-FAM, probe1b-FAM, or probe2a-FAM in table S4) was used as a probe to visualize HCV in cells (the leftmost column of images). The binding of the probe, BG4, and biotin-PDP is indicated as overlapping white regions (indicated by white arrows in the rightmost column of images). As expected, the colocalization was disrupted after treatment with the ASO complementary to the target G4 site (the second, fourth, and sixth rows of images). Moreover, no overlapping white region was observed in the G4-mutated virus (bottom row of images). These results provided direct evidence that G4 ligand binds to the target HCV G4 site under physiological conditions.

**DISCUSSION**

Despite the development of combined treatment with IFN and ribavirin as an antiviral therapy for HCV (62), IFN is poorly tolerated by many people and has limited availability in many countries (63). Moreover, combination therapy has significant side effects (64). Sofosbuvir-based therapy is a new and very effective treatment for HCV infection, but is extremely expensive (65). Hence, there is an urgent need for better anti-HCV regimens with lower costs. Here, we conducted explicit bioinformatics analysis of the HCV genomes of all available genotypes. Remarkably, we first identified a highly conserved G-rich region in the HCV C gene for subtype 1a or 1b from the HCV database and generated a G4-forming sequence between positions +267 and +279 in the J6/JFH1 genome. Because patients with HCV genotype 1 responded poorly to the current therapy (72), we aligned more than 1000 sequences of the C gene for subtype 1a or 1b from the HCV database and generated two representative sequences with high potential to form stable G4 RNAs. Various assays have been used to confirm the formation of G4 RNA. A 1H NMR study revealed far fewer imino peaks in the G4 region for the longer RNA (fig. S10). This analysis, together with the secondary structure prediction (figs. S8 and S9), may suggest a competition between stable stem-loop structures and the proposed G4 structures. Hence, we propose that a target genome sequence does not form a very stable G4 structure under native conditions but rather is induced to refold into a G4 by the presence of a G4 ligand. Further studies confirmed the strong interactions between HCV G4 RNAs and G4 ligands in the presence of potassium at physiological concentrations. Although it has been reported that TMPyP4 might destabilize, rather than stabilize, G4 RNAs (73), this compound acts specifically on target HCV G4 RNAs and shows a much weaker binding to the G4-mutated RNAs.

It has been demonstrated that stable G4 RNA motifs in 5′UTRs might block the translation initiation of proteins in mammalian cells (35). A previous study also demonstrated the suppression of gene expression through G4 RNA formation in several open reading frames encoded within the *Escherichia coli* genome (74). However, in this study, G4 RNAs only inhibited the translation of specific proteins. During the life cycle of HCV, the positive-sense RNA genome is replicated through an RNA intermediate (39). The RNA stop assay in the present study revealed that this specific G4 RNA formation in template RNA led to the inhibition of RNA-dependent RNA synthesis, suggesting the direct inhibition of viral genomic replication through G4 ligands. Moreover, the expression of EGF-containing HCV G-rich RNAs decreased significantly after treatment with G4 ligands, whereas constructs containing G4-disruptive mutations (G to A) were relatively insensitive to G4 ligands. In conjunction with the full-length C gene expression assay, we propose that the expression level of the HCV C gene could be down-regulated through selective G4 RNA formation.

On the basis of these new findings, we further evaluated the antiviral activities of G4 ligands. Here, we first revealed that G4 ligands inhibit intracellular HCV replication (genotype 1a, 1b, or 2a) at both RNA and protein levels. We also confirmed that the specific cellular target of active G4 ligands was HCV G-rich RNA, using an inactive analog as a negative control. The results demonstrated that TMPyP4 efficiently inhibited the expression of a full-length HCV C gene on an engineered plasmid. TMPyP4 further suppressed intracellular HCV (genotype 1a, 1b, or 2a) levels in infected Huh-7.5.1 cells. In contrast to TMPyP4, much alleviated effects in all these studies could be characterized for TMPyP2. Because TMPyP2 is a bad ligand for the binding and stabilization of G4 DNAs (38), these results suggested that the action mode of TMPyP4 in the inhibition of HCV replication might be mediated through binding to target HCV G4 RNA.

To further validate the cellular target for G4 ligand, we mutated the G4-forming sequence between positions +267 and +279 in the J6/JFH1 C gene. The enrichment of G4 RNA in the pull-down extracts of J6/JFH1 was further demonstrated through CD and RT-qPCR determination of RNA recovered from beads. In contrast, almost no RNA.
was recovered from the beads for J6/JFH1-G4-Mut RNA. Together, these results indicate that G4 RNA on the HCV C gene could be selectively recognized and targeted by the G4 ligand. Because the G4-mutated virus survived and reproduced in Huh-7.5.1 cells, this mutant virus could be an ideal model for examining the on-target specificity of the G4 ligand. Comparative analysis of the antiviral activities of the G4 ligand was performed through RT-qPCR and Western blot analyses, and the results showed a significant decrease toward the G4-mutated virus. We hypothesized that G4 RNA formation in the HCV C gene could lead to viral mRNA release from ribosomes due to the steric hindrance of the stable structure.

We further examined the G4 structures in HCV-infected Huh-7.5.1 cells using indirect immunofluorescence microscopy, and clear overlapping regions of BG4, HCV probe, and biotin-PDP immunofluorescence indicated G4 formation in the full HCV genome under physiological conditions. We also characterized the competition between ASO and BG4 (also biotin-PDP) for binding to the target G4 site. For G4-mutated virus, almost no binding between the probe and BG4 (or biotin-PDP) was observed in the cytoplasm of cells. Together, these findings suggest that HCV G4 RNA in the C gene could serve as a new cellular target for anti-HCV drug development.

MATERIALS AND METHODS

Experimental design

There is an urgent need for better anti-HCV regimens with lower costs. Here, our bioinformatics analysis reveals a highly conserved G-rich consensus sequence within the HCV C gene, despite the high genetic variability of riboviruses; we further show using various methods that the HCV C consensus sequences can fold into unimolecular G4 RNA structures both in vitro and under physiological conditions. We then demonstrate that G4 ligands effectively inhibit intracellular HCV replication (genotype 1a, 1b, or 2a) and further validate the specific cellular target of G4 ligands to be HCV G-rich RNA. Ultimately, the stabilization of G4 RNA in the HCV genome represents a promising new strategy for anti-HCV drug development.

Synthesis

Compound PDP and biotin-PDP were synthesized according to a previously described procedure (37).

Bioinformatics analysis

Sequence conservation analysis was conducted using Molecular Evolutionary Genetics Analysis (MEGA) software (version 6.0; available at www.megasoftware.net). The multiple sequence alignment was also carried out with MEGA 6.0 software. HCV sequences were retrieved from the HCV database (www.hcv.lanl.gov/), and the graphical representation of the aligned sequences was generated using the WebLogo software program (version 2.8.2; http://weblogo.berkeley.edu/).

Gel electrophoresis

See the Supplementary Materials for a more detailed protocol.

NMR spectroscopy

$^1$H NMR spectra were recorded at 298 K using an 800-MHz Bruker Avance DRX-800 spectrometer equipped with a cryogenic triple-resonance inverse automatic tuning and matching probe. Water suppression was achieved using the excitation sculpting method. The RNA samples were dissolved in 10 mM phosphate-buffered saline (PBS) (pH 7.0) containing 100 mM KCl and 10% D$_2$O at a final concentration of 0.5 mM. The samples were annealed after heating at 90°C for 4.0 min and slowly cooled to 25°C. All experiments were performed using a diffusion time ($T_{1D}$) of 100 ms, an eddy current recovery time ($T_R$) of 50 ms, and a relaxation delay ($T_E$) of 2 s.

CD studies

See the Supplementary Materials for a more detailed protocol.

CD melting studies

G4 ligands were dissolved as a 10 mM stock solution in DMSO. See the Supplementary Materials for a more detailed protocol.

FRET kinetic assay

Dual-labeled G4 RNA probe (RNA1a-dual or RNA1b-dual) containing a donor fluorophore (FAM) and an acceptor fluorophore (TAMRA) was used. The probe samples (at a final concentration of 200 nM) were prepared in 10 mM tris-HCl buffer (pH 7.0) (100 mM KCl) containing different amounts of PDP, and equilibrated at 4°C overnight. For the FRET kinetic assays, the ASO sample (ASO-1a or ASO-1b at a final concentration of 2.0 μM) was mixed at t = 0. Fluorescence detection was conducted at 25°C in kinetics mode. The same LS55 spectrometer was used with a 1-cm path length cell. The excitation and emission wavelengths were set to 494 and 580 nm, respectively.

RNA stop assay

3D$^{pol}$ was a gift from P. Gong (Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China). The assay was performed as described previously (40). P15 (300 nM) and template RNA (600 nM) were annealed in a reaction buffer [50 mM Heps, 20 mM NaCl, 1.0 mM KCl, 5 mM MgCl$_2$, and 4 mM DTT (pH 7.0)] at 25°C after heating at 85°C for 2.0 min and slowly cooled to 4°C. Subsequently, NTPs (final concentration, 200 μM) and 3D$^{pol}$ (0.02 mg/reaction) were added, and the reactions were performed at 33°C for 20 min. The reactions were stopped after adding an equal volume of stop buffer (95% formamide) and heated at 90°C for 4.0 min. The products were loaded and separated on 20% denaturing polyacrylamide gels. Finally, the gels were scanned by a Pharos FX Molecular Imager (Bio-Rad) operated in fluorescence mode.

Cell lines

Human embryonic kidney (HEK) 293 cells and Huh-7.5.1 cells were cultured under standard culture conditions.

EGFP expression repression through HCV G4 RNA stabilization

HCV G-rich sequences were cloned into the pEGFP-C1 vector by PCR with the following primer sets. The forward primer was designed to include a pendant 5’ segment comprising an Nhe I cleavage site and an HCV G-rich sequence, along with the 3’ region perfectly matching the N terminus of EGFP in frame. The reverse primer was designed to have an Hind III site at the 5’ end and a 3’ region, perfectly matching the C terminus of EGFP. Plasmid pEGFP-C1 (Clontech) was used as PCR template. These two primers (C1-1a-F and C1-R) were used to amplify the 758-bp fragment. The fragment was digested with Nhe I and the results showed a significant decrease toward the G4-mutated virus. The samples were annealed after heating at 90°C for 4.0 min and slowly cooled to 25°C. All experiments were performed using a diffusion time ($T_{1D}$) of 100 ms, an eddy current recovery time ($T_R$) of 50 ms, and a relaxation delay ($T_E$) of 2 s.

CD studies

See the Supplementary Materials for a more detailed protocol.

CD melting studies

G4 ligands were dissolved as a 10 mM stock solution in DMSO. See the Supplementary Materials for a more detailed protocol.

FRET kinetic assay

Dual-labeled G4 RNA probe (RNA1a-dual or RNA1b-dual) containing a donor fluorophore (FAM) and an acceptor fluorophore (TAMRA) was used. The probe samples (at a final concentration of 200 nM) were prepared in 10 mM tris-HCl buffer (pH 7.0) (100 mM KCl) containing different amounts of PDP, and equilibrated at 4°C overnight. For the FRET kinetic assays, the ASO sample (ASO-1a or ASO-1b at a final concentration of 2.0 μM) was mixed at t = 0. Fluorescence detection was conducted at 25°C in kinetics mode. The same LS55 spectrometer was used with a 1-cm path length cell. The excitation and emission wavelengths were set to 494 and 580 nm, respectively.

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and Hind III, and subcloned into the same restriction sites of pEGFP-C1 vector to generate the plasmid construct pEGFP-C1-1a. pEGFP-C1-1b was prepared using the forward primer (C1-1b-F), together with the same reverse primer (C1-R). The primer pair sets (C1-1aMut-F, C1-R and C1-1bMut-F, C1-R) were used to generate pEGFP-C1-1aMut and pEGFP-C1-1bMut. All constructs were confirmed through sequencing. HEK293 cells were transfected with the plasmid using Lipofectamine 2000 (Invitrogen) at a 1:2 ratio (~500 ng of plasmid/1 μl of reagent) and were subsequently treated for 48 hours with different amounts of freshly dissolved G4 ligands. Subsequently, the cells were washed with PBS and fixed using 4% paraformaldehyde at 25°C for 15 min. The cells were washed three times with PBS for 5.0 min each and permeabilized with 0.5% Triton X-100 at 4°C for 10 min. Coverslips with the cells were then mounted using 50% glycerol in PBS and observed using a laser scanning confocal microscope (Leica TCS SP2). The excitation laser used for EGFP was 488 nm; the emission of green fluorescence was collected using the 505- to 530-nm band-pass filter. A laser diode (excitation, 405 nm) and a band-pass filter (420 to 480 nm) were used to record the fluorescence of Hoechst 33258 (blue). Flow cytometry was conducted on a flow cytometer (Coulter XL). Statistical analysis was performed using EXPO32 v1.2 Analysis software (Beckman Coulter).

Construction of a plasmid carrying the G4-mutated C gene

pcDNA3.1-N-Flag vector (75) was a gift from H. Wang (Wuhan Institute of Virology, Chinese Academy of Sciences). The G4-mutated C gene was chemically synthesized at Invitrogen, and the sequence in the G-rich region (positions +265 to +279) was mutated (GAGGCT-TGCGCTTG to GAAGGCGTATTTT). Two primers (1b core G4-Mut F and 1b core G4-Mut R) were used to amplify the 595-bp fragment. The fragment was digested with Bam HI and Not I and subcloned into the same restriction sites of pcDNA3.1-N-Flag vector to generate the plasmid construct 1b core G4-Mut.

Western blot analysis to detect HCV C gene expression

For this assay, total proteins were extracted using a radioimmunoprecipitation assay lysis buffer and quantitated using bicinchoninic acid protein assay. The lysates were mixed with an equal volume of 2× loading buffer and boiled for 5 min. Then, 50 μg of proteins was subjected to electrophoresis on 10% SDS-PAGE gels (1.0 mm thick × 15 cm long), along with 15 μl of Kaleidoscope protein standards (Bio-Rad), and transferred to polyvinylidene difluoride membranes (Millipore). After blocking with 5.0% bovine serum albumin in tris-buffered saline and Tween 20, target proteins on membranes were probed at 37°C for 1 hour with anti–HCV Core 1b antibody (1:1000 dilution; Abcam). β-Actin was used as a loading control for analysis and was probed by monoclonal mouse anti-actin antibody (1:1000 dilution; Abcam). After the final wash, the signal was developed with corresponding horseradish peroxidase-conjugated secondary antibody (1:5000; Proteintech). Finally, protein bands were visualized on an x-ray film using enhanced chemiluminescence reagents (Millipore).

Antiviral activity assay

Genotype 2a JFH1 HCVcc (cell culture–derived HCV) stock was generated as previously described (46, 53). Genotype 1a/2a chimera (H77/ JFH1 chimera) was a gift from Z. Qi (Department of Microbiology, Second Military Medical University, Shanghai, China), and genotype 1b/2a chimera (Con1/JFH1 chimera) HCVcc stock (50) was a gift from X. Chen (Wuhan Institute of Virology, Chinese Academy of Sciences). The infectious titers were determined after immunofluorescence staining against HCV NS5A (anti-NS5A polyclonal antibody, a gift from J. Zhong, Unit of Viral Hepatitis, Institut Pasteur of Shanghai, China). Daclatasvir and IFN-α were used as positive controls. The cells were cultured in complete Dulbecco’s minimum essential medium (DMEM) and passaged every 3 to 5 days. The corresponding supernatants of the HCV-containing cell culture were collected and filtered through a 0.45-μm filter membrane (Thomas Scientific), and the filtrates were concentrated with a Vivaspin (100-kD cutoff; Millipore). The concentrated virus-containing medium was loaded onto a 20% sucrose cushion in an ultracentrifuge tube, and the cell culture–adapted virus particles were purified using ultracentrifugation (4°C for 4 hours) at 28,000 rpm (SW48 rotor; Beckman Coulter). The resulting pellets were resuspended in 0.5 ml of complete medium, and naïve Huh-7.5.1 cells were infected with the diluted viruses at 3.0 multiplicity of infection for 4 hours. After infection, the cells were washed with PBS to remove unbound virus. Fresh complete DMEM was added, and HCV-infected cells were treated for 72 hours with different amounts of freshly dissolved G4 ligands. For RT-qPCR, cells were lysed with TRIzol reagent (Invitrogen), and total RNA was extracted and purified according to the manufacturer’s instructions. The purified RNAs were dissolved in diethyl pyrocarbonate–treated water and spectrophotometrically quantitated. Reverse transcription of RNA to synthesis cDNA was performed with a ReverTra Ace-α-Kit (TOYOBO). RT-qPCR was conducted in optical tubes in a 96-well microtitre plate (Applied Biosystems) with an ABI PRISM 7700 Sequence Detector Systems (Applied Biosystems). SYBR Green Realtime PCR Master Mix-Plus Kit (TOYOBO) was used, and fluorescence signals were generated and recorded during each PCR cycle. HCV RNA levels at the C gene and 5′ UTR were quantitated, and the GAPDH mRNA of host cells was used as a control. The following primer sets were used in this study: JFH1 Core-F/R, H77 Core-F/Core-R, Con1 Core-F/Core-R, 5′UTR-F/R, and GAPDH-F/R (table S4). Western blot analysis was conducted using the above procedure described for HCV C gene expression, and the target proteins were probed with anti–HCV Core 1a or 1b antibody (1:1000 dilution; Abcam) or anti-NS3 antibody (1:1000 dilution; Abcam).

Construction of G4-mutated plasmids and G4-mutated virus

To construct a target plasmid containing a G4-mutated HCV C gene with multiple point mutations, we used a starting plasmid vector pJ6/JFH1 (a gift from Z. Qi) (56). The multiple point mutations at nucleotides 267, 269, 275, 278, and 279, respectively (GAGG-GAGGCT-TGCGCTTG to GAAGGCGTATTTT), were introduced after amplifying DNA fragments containing the mutations. Point mutations were created through OE-PCR of the region between nucleotides 267, 269, 275, 278, and 279, respectively (GAGG-GAGGCT-TGCGCTTG to GAAGGCGTATTTT). Two primer sets (forward primer in upstream region (J6 up F), reverse primer in downstream region (J6 down R)) were used. The target fragment was digested with Eco RI and Kpn I and subcloned into the same restriction sites of the pJ6/JFH1 vector to generate the plasmid construct pJ6/JFH1–G4-Mut, which was further confirmed by sequencing.

In vitro transcription and activity assay

In vitro transcription reactions were performed according to the manufacturer’s instructions in the MEGAscript T7 Transcription Kit.
with 20 hundred microliters of each sonicated RNA sample was incubated with a pulse of 30 s on/30 s off, to an average of 100 bp. Four sonicators (300 W; Ningbo Scientz Biotechnology) for 2 hours at high

enriched DNA was diluted to 1 ng/μl. Input reverse-transcribed genomic DNA or reverse-transcribed G4-RT-qPCR validation of G4-specific enrichment

cell with a 1.0-cm path length. The eluted RNA swas purified through spin-drop chromatography and redissolved in 10 mM tris-HCl buffer (pH 7.0) containing 100 mM KCl. CD experiments were performed using a quartz

amide [2.5 μl of 0.2 M EDTA (pH 8.0) and 47.5 μl of formamide] at 90°C for 5 min. The eluted RNAs were purified through spin-column chromatography and redissolved in 10 mM tris-HCl buffer (pH 7.0) containing 100 mM KCl. CD experiments were performed using a quartz cell with a 1.0-cm path length.

Pull-down assay

In vitro–transcribed RNAs (400 μg) were incubated in 10 mM tris-HCl (pH 7.0) buffer containing 100 mM KCl in the presence or absence of 5.0 μM biotin-PDP and then sheared with the SB-5200 DTD sonicator (300 W; Ningbo Scientz Biotechnology) for 2 hours at high power with a pulse of 30 s on/30 s off, to an average of 100 bp. Four hundred microliters of each sonicated RNA sample was incubated with 20 μl of hydrophilic streptavidin magnetic beads (S1420S, New

Brazil Laboratory) for 1 hour at 37°C. The separated magnetic beads were subsequently incubated with 10 mM EDTA and 95% formamide [2.5 μl of 0.2 M EDTA (pH 8.0) and 47.5 μl of formamide] at 90°C for 5 min. The eluted RNAs were purified through spin-column chromatography and redissolved in 10 mM tris-HCl buffer (pH 7.0) containing 100 mM KCl. CD experiments were performed using a quartz cell with a 1.0-cm path length.

RT-qPCR validation of G4-specific enrichment

Input reverse-transcribed genomic DNA or reverse-transcribed G4-enriched DNA was diluted to 1 ng/μl, and 1 μl was used in triplicate 20-μl qPCRs containing 1× LightCycler 480 SYBR Green I Master (Roche Diagnostics), 0.5 μM forward and reverse primers, and water. The reactions were run on a Roche LightCycler 480 II under standard cycling conditions. The G4 locus–associated primers included G4-fwd and G4-rev, and the fold enrichment was calculated as $2^{ΔC_{t}} = C_{t} (G4 enriched) − C_{t} (input)$.

ASO treatment enhances viral replication

HCV-infected Huh-7.5.1 cells (5 × 10⁵) were transfected with either a 19-mer ASO (AS-RNA1a, AS-RNA1b, AS-RNA2a, or AS-Gmut2a), a 19-mer mutant ASO (AS-mutant1a, AS-mutant1b, or AS-mutant2a), or a random 19-mer oligonucleotide (oligo control), each at a final concentration of 100 nM, using Oligofectamine reagent (Invitrogen) according to the manufacturer's protocol. At 48 hours after transfection, the cells were lysed with TRIzol reagent, and total RNA was determined through RT-qPCR. In these studies, the HCV RNA levels were determined relative to the transcription of GAPDH in host cells. The primer sets (UTR and GAPDH) were the same as those for antiviral activity assay. Values for the "ASO" or "mutant ASO" groups were calculated relative to the "oligo control" value.

Fluorescent imaging to visualize HCV G4 RNAs in cells

The expression and purification of the G4-specific antibody BG4 were performed according to the previously described protocol (11).

Huh-7.5.1 cells were transfected with FAM-labeled oligomers or full-length HCV genomes using Oligofectamine reagent (Invitrogen) and were treated with or without 2.5 μM biotin-PDP for 4 hours. The demonstration of full-length HCV genomes inside cells was confirmed through fluorescence in situ hybridization using a FAM-labeled ASO complementary to a sequence upstream of the target G4 site. Cells on glass coverslips were washed with a culture medium and PBS, fixed, permeabilized, and blocked following a previous protocol (60). Specifically, cells were permeabilized in 0.02% Triton X-100/PBS buffer for 15 min at 4°C (76). Subsequently, immunofluorescence was measured. Briefly, the cells were incubated at 37°C with BG4 (4 μg/ml), followed by anti-Flag antibody (1:500 dilution; no. 2368, Cell Signaling Technology) and anti-rabbit rhodamine-conjugated antibody (1:500 dilution; R-6394, Life Technologies). APC conjugated streptavidin (1:500 dilution; S686, Life Technologies) was used to demonstrate biotin-PDP. The excitation laser used for the FAM group was 488 nm. Rhodamine was excited with a 568-nm diode laser and detected using a 590-nm long-pass filter. APC was excited at 639 nm and detected with a 647-nm long-pass filter.

Statistical analysis

The experimental data were analyzed with Student’s t test using the SPSS 17.0 software. *P < 0.05 was considered statistically significant.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/2/4/e1501535/DC1

Materials and Methods

Fig. S1. Structural illustration of a G-quartet.

Fig. S2. Conservation analysis of HCV genomes.

Fig. S3. Premade sequence alignment in the central part of the HCV C gene, between positions +294 and +328.

Fig. S4. Premade sequence alignment in the central part of the HCV C gene, between positions +253 and +294.

Fig. S5. Premade sequence alignment in the central part of the HCV C gene, between positions +236 and +294.

Fig. S6. Expansion of the 1H NMR spectra of RNA1a.

Fig. S7. Expansion of the 1H NMR spectra of RNA1b.

Fig. S8. Prediction of the RNA secondary structure of the C gene (subtype 1a) using freenergy minimization.

Fig. S9. Prediction of the RNA secondary structure of the C gene (subtype 1b) using freenergy minimization.

Fig. S10. G4 structure of RNA1a is more stable than that of RNA1b.

Fig. S11. Mapping of the plasmid 24480 (pMO29) and a sequenced portion of this plasmid for RNA1a.

Fig. S12. G4 structure of RNA1a is more stable than that of RNA1b.

Fig. S13. G4 RNA molecules were characterized in the presence of alkali metal ions (K+, Na+, or Li+).

Fig. S14. HCV C4 RNA structures are destabilized through the ASO.

Fig. S15. CD melting curves of HCV G-rich RNAs.

Fig. S16. Influence of different alkali metal ions on the thermal stabilities of HCV G4 RNAs.

Fig. S17. Analysis of concentration-independent melting curves of target HCV RNAs.

Fig. S18. CD melting studies of target HCV RNAs.

Fig. S19. Structures of TMPyP4 and TMPyP2.

Fig. S20. G4 ligand stabilizes target HCV G4 RNAs.

Fig. S21. Little interaction is observed between the G4 ligand and G4-mutated RNAs.

Fig. S22. Schematic depiction of the inhibition of FRET through the binding between PDP and G4 RNA.

Fig. S23. PDP binds to target G4 RNA and inhibits the trap by the corresponding ASO.

Fig. S24. G4 ligand inhibits RNA-dependent RNA synthesis through G4 RNA stabilization.

Fig. S25. Map of the plasmid 24480 (pMO29) and a sequenced portion of this plasmid for verification.

Fig. S26. TMPyP2 does not stabilize G4 RNA for RNA1b.

Fig. S27. G4 ligands do not suppress the expression of the HCV C gene containing a G4-mutated sequence.

Fig. S28. G4 ligands repress the in vitro expression of EGFP through G4 RNA stabilization.

Fig. S29. Structures of TMPyP4 and TMPyP2.

Fig. S30. G4 ligand stabilizes target HCV G4 RNAs.

Fig. S31. Little interaction is observed between the G4 ligand and G4-mutated RNAs.

Fig. S32. Schematic depiction of the inhibition of FRET through the binding between PDP and G4 RNA.

Fig. S33. PDP binds to target G4 RNA and inhibits the trap by the corresponding ASO.

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Fig. S35. Map of the plasmid 24480 (pMO29) and a sequenced portion of this plasmid for verification.

Fig. S36. TMPyP2 does not stabilize G4 RNA for RNA1b.

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Fig. S38. G4 ligands repress the in vitro expression of EGFP through G4 RNA stabilization.
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A highly conserved G-rich consensus sequence in hepatitis C virus core gene represents a new anti-hepatitis C target

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