Replication of hepatitis C virus (HCV) RNA is catalyzed by the virally encoded RNA-dependent RNA polymerase NS5B. It is believed that the viral polymerase utilizes a de novo or primer-independent mechanism for initiation of RNA synthesis. Our previous work has shown that dinucleotides were efficient initiation molecules for NS5B in vitro (W. Zhong, E. Ferrari, C. A. Lesburg, D. Maag, S. K. Ghosh, C. E. Cameron, J. Y. Lau, and Z. Hong. J. Virol. 74:9134-9143, 2000). In this study, we further demonstrated that dinucleotide analogues could serve as inhibitors of de novo initiation of RNA synthesis directed by HCV NS5B. Both mononucleotide- and dinucleotide-initiated RNA syntheses were affected by dinucleotide analogues. The presence of the 5'-phosphate group in the dinucleotide compounds was required for efficient inhibition of de novo initiation. Optimal inhibitory activity also appeared to be dependent on the base-pairing potential between the compounds and the template terminal bases. Because the initiation process is a rate-limiting step in viral RNA replication, inhibitors that interfere with the initiation process will have advantages in suppressing virus replication. The use of dinucleotide analogues as inhibitor molecules to target viral replication initiation represents a novel approach to antiviral interference.

Hepatitis C virus (HCV) infection is an important public health problem worldwide and is recognized as the major cause of non-A, non-B hepatitis. It is estimated that HCV affects 4 million people in the United States, 8 million people in Europe and Japan, and, collectively, 170 million people worldwide (22, 24). Although HCV infection resolves in some cases, the virus establishes chronic infection in up to 80% of the infected individuals and persists for decades. It is estimated that about 20% of these infected individuals will go on to develop cirrhosis, and 1 to 5% will develop liver failure and hepatocellular carcinoma (23, 24, 26). Chronic hepatitis C is the leading cause of chronic liver disease and the leading indicator for liver transplantation in the United States. The Centers for Disease Control and Prevention estimate that hepatitis C currently is responsible for approximately 8,000 to 10,000 deaths in the United States annually. This number is projected to increase significantly over the next decade. Currently, there is no vaccine for HCV infection due to the high degree of heterogeneity of this virus.

The objectives for the treatment of chronic hepatitis C are to achieve complete and sustained clearance of HCV RNA in serum and normalization of serum alanine aminotransferase levels. In the absence of a prophylactic vaccine or a highly specific antiviral agent, treatment options for chronically infected individuals are limited. The current treatment options for chronic hepatitis C include (pegalated) alpha interferon (IFN-α) monotherapy and (pegalated) IFN-α-ribavirin combination therapy, with sustained virological response rates of between 10 and 60% (4, 7, 15-17, 20, 21). Clearly, more effective and direct antiviral interventions are necessary for further prevention and treatment of the life-threatening complications caused by HCV infection. Efforts to identify and develop highly specific and potent HCV inhibitors have intensified recently. Investigators have targeted all regions of the HCV genome and virally encoded replication enzymes for potential therapeutic discovery.

HCV is a positive-strand RNA virus belonging to the family Flaviviridae (3). This virus family also contains about 40 flaviviruses that are associated with human diseases, including the dengue fever viruses, yellow fever viruses and Japanese encephalitis virus, as well as pestiviruses, whose infection of domesticated livestock can cause significant economic losses worldwide. Like other RNA viruses, a virally encoded replication enzyme, RNA-dependent RNA polymerase (RdRp), plays a central role in viral RNA replication of HCV and other members of the family Flaviviridae. In the case of HCV, the replication protein is termed “NS5B” (nonstructural protein 5B) (2, 5, 6, 12, 13). RdRp proteins are the key components of the viral replicase complexes and therefore serve as attractive targets for antiviral development (1, 8, 9, 11).

It is generally believed that HCV RNA replication is initiated by NS5B RdRp via a de novo or primer-independent mechanism (10, 14, 18, 19, 25, 28). Our previous observations suggest that dinucleotides can be used more efficiently as initiation molecules by HCV NS5B than mononucleotides in the in vitro RNA replication assay (27). It is thus conceivable that dinucleotide analogues may have potential to inhibit initiation of HCV RNA replication. Because the initiation process is considered to be the rate-limiting step in viral RNA replication, inhibitors that interfere with the initiation process will have advantages in suppressing virus replication. The use of dinucleotide analogues as inhibitor molecules to target the initiation step of viral RNA synthesis represents a novel approach to antiviral interference.
FIG. 1. Chemical synthesis schemes of dinucleotide analogues I (A) and II (B).
FIG. 2. Inhibition of HCV NS5B-catalyzed de novo initiation of RNA synthesis by dinucleotide analogues. (A) Structures of dinucleotide analogues I and II. (B) Inhibition of NS5B-directed de novo initiation of RNA synthesis. A short synthetic RNA (5’AAAAAAAGC 3’) was used as a template in the de novo initiation assay. GTP and radiolabeled CTP were used as the initiating and the elongating nucleotides, respectively. A standard reaction mixture in a total volume of 20 μl contained 50 mM HEPES (pH 7.5), 10 mM β-mercaptoethanol (β-ME), 10 mM MgCl₂, 5 μM RNA template, 100 μM GTP, 100 μM CTP–10 μCi of [α-33P]CTP, 1 μM HCV NS5B, and increasing concentrations (0 to 300 μM) of the analogue compounds. The reaction mixture was incubated at 30°C for 30 min, and the RNA products were resolved on a 25% polyacrylamide gel electrophoresis–6 M urea–Tris-borate-EDTA gel prior to PhosphorImaging analysis. Compounds I and II are shown in lanes 2 to 6 and 7 to 11, respectively. The labeled dinucleotide product (pppGpC) is indicated on the left.
Synthesis of dinucleotide compounds. We initiated this study by synthesizing two dinucleotide analogues (I and II) (Fig. 1) that contained a guanine base at the 5' end and a cytosine base at the 3' end. Modifications were introduced in the sugar moieties (2'- or 3'-O-methyl) and the linker (phosphorothioate) for improvement of compound stability. In addition, a 5'-phosphate group was added to further mimic the natural dinucleotides. These compounds were guanine-cytosine analogues (pGpsC).

The dinucleotide compounds were synthesized on solid support at a 100-mol scale with an Applied Biosystems 394 DNA/RNA synthesizer. For dinucleotide I, 3'-dC-CPG 500 (Glen Research) (compound 1 in Fig. 1A) was used as the starting material. For dinucleotide II, 5'-O-dimethoxytrityl-N4-benzoyl-3'-O-methylcytidine-2'-O-[2-cyanoethyl-(N,N-diisopropyl)]phosphoramidite (compound 7 in Fig. 1B) was linked to universal solid support. The synthesis of the dinucleotide compounds was carried out with the following common steps (Fig. 1): (i) detritylation (3% dichloroacetic acid [DCA] in CH2Cl2 for 3 min); (ii) coupling with the next monomer, 5'-O-dimethoxytrityl-N-isobutyryl-2'-O-methylguanosine-3'-O-[2-cyanoethyl)-(N,N-diisopropyl)]phosphoramidite (compound 2 in Fig. 1A); (iii) oxidation using Beaucage reagent to the phosphorothioate; (iv) detritylation; (v) coupling of the 5'-phosphate group using chemical phosphorylation reagent 4 (compound 4 in Fig. 1A) (Glen Research); and (vi) oxidation using iodine-water to 5'-phosphate.

The chemical phosphorylation reagent used for addition of the 5'-phosphate had a terminal dimethoxytrityl (DMTr) group, which was utilized to facilitate reverse-phase high-performance liquid chromatography (HPLC) purification. After completion of the synthesis, dinucleotide I was treated with NH4OH at 60°C for 22 h, while dinucleotide II was treated at 80°C for 8 h. This NH4OH treatment cleaved dinucleotides from solid support and removed protecting groups from nucleobases and internucleoside phosphate linkage. The compounds were then purified with C18 columns (Waters). The dilute trifluoroacetic acid solution was used as a mobile phase to remove the DMTr protection group. The purified dinucleotide compounds were characterized by mass spectrum (I, M+H+ = 683.62; II, M+H+ = 713.42). HPLC purity was more than 85%, with the single largest impurity not more than 3%.

Dinucleotide analogues inhibited de novo initiation of RNA synthesis directed by HCV NS5B. To determine whether the synthesized dinucleotide compounds (pGpsC analogues) can inhibit initiation of RNA replication by HCV NS5B, a de novo initiation assay was performed, which used a small synthetic 10-mer RNA (5' AAAAAAAAGC 3') as the template. In this assay, GTP and radiolabeled CTP were used as the initiating and elongating nucleotides, respectively.

![Diagram of pppGpC](http://aac.asm.org/Downloaded)
The 3′-terminal sequence of negative-strand HCV RNA (−GC 3′) serves as a template for multiple rounds of positive-strand RNA synthesis. (ii) The dinucleotide compounds synthesized mimick guanine-cytosine analogues. It has been demonstrated previously that GTP is an efficient initiation molecule for HCV NS5B in a number of assays (14, 19, 25, 28). As shown in Fig. 2, both dinucleotide analogues I (lanes 2 to 6) and II (lanes 7 to 11) had an inhibitory effect on NS5B-directed de novo initiation, with 50% inhibitory concentrations (IC50s) of 20 and 65 μM, respectively. This result indicated that the dinucleotide compounds were able to efficiently compete with the initiating nucleotide GTP for binding to the polymerase protein and were able to inhibit the initiation of RNA synthesis.

The difference between these two dinucleotide analogues was that one contains a 3′-O-methyl group at the C3′ of the second ribose moiety (II), while the other contains a hydrogen (3′-H) at the same position (I). The relatively weaker activity for analogue II (higher IC50) implied that addition of a larger group at the 3′ position reduced the binding affinity to the NS5B polymerase and thus competed less effectively with the initiating GTP.

The 5′-phosphate group was important for the inhibitory activity of the dinucleotide analogues. Both analogues tested in Fig. 2 contained a phosphate group at the 5′ terminus. This was based on the assumption that the phosphate group might contribute to the binding affinity with NS5B polymerase. To demonstrate that such a phosphate group was indeed important for the inhibitory activity of the dinucleotide compounds, a third analogue (III) was tested in the same replication initiation assay. Analogue III possessed a similar structure to analogues I and II, except the 5′-phosphate group was deleted. As shown in Fig. 3, analogue III lost its inhibitory activity on de novo initiation of RNA synthesis, with an IC50 of >200 μM (com-
pare lanes 6 and 7 with lanes 2 to 5). This result suggested that the 5′-phosphate group of the dinucleotide analogues played an important role in the interaction with NS5B protein, the lack of which resulted in loss of binding affinity required for efficient competition with the initiating GTP.

Optimal inhibitory activity of the dinucleotide analogues was dependent on the base-pairing potential between the analogues and the template 3′-terminal bases. To determine whether the inhibition of de novo initiation by dinucleotide analogues was template sequence specific and whether base-pairing potential between the compounds and the template RNA played a role in stabilizing the compound-NS5B interaction, de novo initiation assays were performed with two separate RNA templates that contained different 3′-terminal bases (Fig. 4). One template (5′ AAAAAAAAGU 3′) used ATP as the initiating nucleotide, whereas the other (5′ AAAAAAAAGC 3′) used GTP as the initiating nucleotide. The radiolabeled dinucleotide products generated from the de novo initiation assays were pppApC and pppGpC, respectively. The inhibitory effects of analogue I (which showed the highest activity in previous assays) on RNA initiation from the two RNA templates were compared. As shown in Fig. 4, this compound showed a more profound effect on GTP-initiated RNA synthesis (IC50 of 20 μM) than on ATP-initiated RNA synthesis (IC50 of 90 μM). This result, together with the fact that the dinucleotide compound tested was a guanine-cytosine (GC) analogue, suggested that the base-pairing potential between the compound and the 3′-terminal bases of the template RNA played a positive role in maximizing the inhibitory activity on de novo initiation of RNA synthesis.

Inhibition of RNA synthesis initiated by dinucleotide primers. Prior assays in determining the compounds’ activity were performed using mononucleotide (GTP or ATP) as the initiating molecule. We have shown previously that dinucleotides were more efficient as initiating molecules than mononucleotide in NS5B-directed RNA replication assays (27). It would be interesting to determine whether the dinucleotide analogues were capable of inhibiting RNA synthesis initiated from dinucleotide primers. To this end, radiolabeled dinucleotide primer ([33P]GpC) was used to prime RNA synthesis (UTP incorporations) from the template RNA 5′ AAAAAAAAGC 3′ in the presence of increasing concentrations of compound I. As shown in Fig. 5, the dinucleotide analogue was also active in inhibiting pGpC-initiated RNA synthesis (multiple rounds of UMP incorporations), with an IC50 of approximately 50 μM (lanes 1 to 6). This result confirmed that the dinucleotide inhibitors were capable of competing with dinucleotide initiating molecules and inhibiting RNA replication. The apparent reduction in the potency of the compound likely reflected the tighter binding between the enzyme and dinucleotide initiating molecule, resulting in less efficient competition by the compound.

Initiation of RNA synthesis by viral replication machinery represents a key step in the replication cycle of RNA viruses. It is generally believed that initiation is a rate-limiting step in viral RNA replication and that therapeutic interference at this step will effectively reduce the level of virus replication. So far, compounds specifically targeting the initiation process of viral RNA replication have not been thoroughly documented. In this study, we showed that dinucleotide compounds could serve as potential inhibitors of de novo initiation of RNA synthesis.
directed by HCV NS5B polymerase. This observation was in accordance with our previous results showing that dinucleotides were efficient initiating molecules for HCV NS5B-directed RNA replication. The use of dinucleotide analogues as inhibitors to target the initiation step of viral RNA synthesis represents a novel approach to antiviral interference.

Despite the successful confirmation of the concept that dinucleotide analogues are effective initiators of de novo RNA replication, significant challenges remain before further development of this class of compounds for direct therapeutic application can be considered. Dinucleotide molecules, especially RNA, are generally unstable. The intramolecular nucleophilic attack by the 2′-hydroxyl group in the sugar moiety on the adjacent phosphate bond causes the degradation of the dinucleotides to mononucleotides and even further to nucleosides. Dinucleotides can also be hydrolyzed by water and other nucleophiles present in the biological system. In addition, the 5′-phosphate dinucleotides, which were shown to be more effective in inhibiting de novo initiation of RNA replication, are even less stable because of the presence of the additional phosphate group. Furthermore, the polyanionic nature of the dinucleotide molecules strongly hampers the penetration of such compounds through cell membrane due to the high lipophilicity on the membrane surface. Finally, dinucleotide molecules can also bind to other nucleic acids through hydrogen bonds, which may result in a loss of selectivity for a predetermined target in vivo.

The pGipsC type of dinucleotide analogues used in this study contained a phosphorothioate linker. Such linker is believed to enhance the stability of the dinucleotide compounds over the natural nucleic acid. For the same purpose, the 2′-hydroxyl group of the sugar moiety was further modified by methylation so that the 2′-hydroxyl group is not available for intramolecular hydrolysis. Nucleic acids with 2′-O-methyl modifications have been reported to possess improved stability, lipophilicity, and bioavailability, as well as other pharmacological properties. The physicochemical and pharmacological features of the dinucleotide compounds can be further improved by modifications of the phosphate groups in reducing their anionic character and, in the mean time, increasing lipophilicity. In this case, the various conjugation strategies used for improving properties of oligonucleotide compounds may be applied.

Despite all of these challenges, the demonstration that dinucleotide analogues can effectively inhibit de novo initiation of RNA replication by HCV NS5B confirms the proof of principle for this novel antiviral approach. With future advancements in addressing the outstanding issues related to the dinucleotide compounds, this class of compounds may eventually reach a stage of development for therapy against important viral diseases.

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REFERENCES
RNA synthesis catalyzed by HCV RNA-dependent RNA polymerase. Bio-
26. World Health Organization. 1996. Hepatitis C. Seroprevalence of hepati-
349.
Cameron, J. Y. N. Lau, and Z. Hong. 2000. Template/primer requirements
and single nucleotide incorporation by hepatitis C virus nonstructural pro-
tein 5B polymerase. J. Virol. 74:9134–9143.
initiation of RNA synthesis by hepatitis C virus nonstructural protein 5B