Arresting Initiation of Hepatitis C Virus RNA Synthesis Using Heterocyclic Derivatives*

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The hepatitis C virus (HCV) NS5B protein encodes an RNA-dependent RNA polymerase (RdRp), the primary catalytic enzyme of the HCV replicase complex. Recently, two benzo-1,2,4-thiadiazine compounds were shown to be potent, highly specific inhibitors of the genotype 1b HCV RdRp containing a carboxyl-terminal 21 residue truncation (Δ21 HCV RdRp) (Dhanak, D., Dufour, X., Johnston, V. K., Lin-Goerke, J., Darcy, M., Shaw, A. N. G. B., Silverman, C., Gates, A. T., Earnshaw, D. L., Casper, D. J., Kaura, A., Baker, A., Greenwood, C., Gutshall, L. L., Maley, D., DelVecchio, A., Macarron, R., Hofmann, G. A., Alnoah, Z., Cheng, H.-Y., Chan, G., Khandekar, S., Keenan, R. M., and Sarisky, R. T. (2002) J. Biol. Chem. 277, 38322–38327). Compound 4 (C21H21N3O4S) reduces viral replication by virtue of its direct interaction with the viral polymerase rather than by nonspecific titration of nucleic acid template. In this study, we present several lines of evidence to demonstrate that this inhibitor interferes with the initiation step of RNA synthesis rather than acting as an elongation mechanism. Inhibition of initial phosphodiester bond formation occurred regardless of whether replication was initiated by primer-dependent or de novo mechanisms. Filter binding studies using increasing concentrations of compound 4 did not interfere with the ability of Δ21 HCV RdRp to interact with nucleic acid. Furthermore, varying the order of reagent addition in the primer extension assay showed no distinct differences in inhibition profile. Finally, surface plasmon resonance analyses provided evidence that a ternary complex is capable of forming between the RNA template, RdRp, and compound 4. Together, these data suggest that this heterocyclic agent interacts with the apoenzyme, as well as with the RNA-bound form of Δ21 HCV RdRp, and therefore does not directly interfere with the RdRp-RNA interaction to mediate inhibition.

Hepatitis C virus (HCV), a positive strand RNA virus of the Flaviviridae family, is the major etiologic agent of post-transfusion and sporadic non-A, non-B hepatitis (2). HCV causes significant liver disease, cirrhosis and can eventually lead to the development of hepatocellular carcinoma. This disease is of significant concern because more than 2% of the world population is chronically infected with HCV. In infected cells, translation of the viral RNA yields a polyprotein (3–5) that is subsequently cleaved to yield structural proteins required for the assembly of new virus particles, as well as nonstructural enzymes essential for viral replication (6–8).

Nonstructural protein 5B (NS5B) encodes RNA-dependent RNA polymerase (RdRp) activity (9, 10); the catalytic activity associated with this enzyme has been confirmed to be required for infectivity in chimpanzees (11). RdRp initiates RNA synthesis preferentially from the 3′ terminus of the template RNA (12–14), although in vitro it has been shown to lack specificity for viral RNA because it readily utilizes heterologous nonviral templates (9). Like poliovirus (15), the HCV RdRp is capable of initiating viral RNA synthesis in vitro by a primer-dependent mechanism (9, 10, 16). However, Flaviviridae RdRps have also been shown to initiate RNA synthesis by a de novo mechanism (12, 13, 17–19). De novo initiation of virus replication is the likely preferred mechanism for HCV in infected cells (20, 21). Recently, our understanding of HCV replication has been significantly improved by Hong et al. (22), whose work resulted in the identification of a novel β-loop near the catalytic site of the RdRp. This has provided a direct structural basis for de novo initiation and suggests that the HCV RdRp can select against primer extension (22).

Cell-based HCV replicon systems, useful for studies of viral replication and antiviral agents, were developed in which the nonstructural proteins stably replicate subgenomic viral RNA in Huh7 cells (23, 24). In combination with studies using recombinant RdRps, such systems are proving to be invaluable in developing a better insight into the mechanisms of Flaviviridae RNA synthesis. This report describes the mechanism of action studies for the heterocyclic inhibitor of the HCV RdRp, compound 4 (C21H21N3O4S, as described in Ref. 1), based on biochemical and cellular studies.

EXPERIMENTAL PROCEDURES

Protein Purification—HCV NS5B protein was purified as described previously (25).

Heparin RdRp Assay—The RdRp assay was performed as described previously (25), with the following modifications. Briefly, after a 15-min incubation, heparin was added to the reaction at a final concentration of 10 ng/ml. At the same time that heparin was added, 15 μM 3′-ribodeoxy-GTP (3′-rdGTP), compound 1 (C20H19N3O4S) or compound 4 was also added. The reaction was continued for an additional 60 min and then terminated, processed, and analyzed as described previously (1).

Initiation Assays—To specifically examine whether the thiadiazines can abrogate dinucleotide polymerization, the initiation step of replica-

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† The abbreviations used are: HCV, hepatitis C virus; NS5B, nonstructural protein 5B; RdRp, RNA-dependent RNA polymerase; nt, nucleotide(s); SPR, surface plasmon resonance; RU, relative units; PFA, phosphonofomeric acid; TNTase, terminal transferase; 3′-rdGTP; 3′-ribodeoxy-GTP.
tion, a direct initiation assay was established. 100 ng (1 µM) of a 17-mer RNA (5'-GGCAGCAGCCAGCUAAC-3') was utilized as the template, and a 2-fold molar excess of the initiation NTP, either GTP or the dinucleotide 5'-GpU3', and the second NTP, [α-32P]UTP, were used to initiate RNA synthesis in 20 mM Tris-HCl, pH 7.5, 5 mM KC1, 3 mM dithiothreitol, 2.5 mM MgCl2, 1 mM MnCl2, and 0.5% (v/v) Triton X-100. After 1 µM polymerase was added, the reaction was allowed to proceed for 30 min and the total RNA products were analyzed on a 25% polyacrylamide gel containing 7.5 M urea. Reaction products (3-mer) were analyzed by autoradiography.

Additionally, primer-dependent and de novo initiation was measured with a single-stranded RNA template, LE21, that can direct both de novo initiation (which produces a 21 nt product) and primer extension (which produces a 34 nt product). Standard RdRp assays consisted of 2.5 pmol of template LE21 with 50-100 ng of NS5B in 20 µl containing final concentrations of 20 mM sodium glutamate at pH 8.2, 12 mM dithiothreitol, 4 mM MgCl2, 2 mM MnCl2, 0.5% (v/v) Triton X-100, 100 µM GTP, 100 µM ATP, 10 µM UTP, 250 nm [α-32P]CTP (400 Ci/mmol), 10 mM UTP, Amersham Biosciences), and ~0.2 µl of 10 mM NaCl (1 µM HCV RdRp Inhibitors). Preincubation of the reaction mixture with 10 nM HCV NS5B, and 10 nM compound 4 was utilized as a control reaction lacking compound 4 but containing an equivalent amount of Me2SO. Therefore, the percent inhibition of 21 nt product was expressed as percentage relative to that of the control reaction (taken as 0% inhibition). The amount of 34 nt product was also normalized in the same manner.

Filter Binding Assay—In vitro binding reactions were carried out using 5 µM purified NS5B2Δ21 in a 50-µl reaction volume containing 20 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 25 mM KC1, 3 mM dithiothreitol, and 0.05% bovine serum albumin. Reaction mixtures containing increasing amounts of 32P-end-labeled SLD8 RNA (5'-GGCCUUGGGCGCCUGU- CUGAUAGCAGAGACCA-3') were incubated for 15 min at 30 °C and then filtered immediately onto an 0.45 µM filters (Whatman), washed with 1 ml of ice-cold binding buffer, and counted in a scintillation counter. For studies using compound 4, two distinct preincubation protocols were followed. For one study, compound 4 was preincubated with polymerase and buffer for 15 min prior to the addition of RNA. Alternatively, the polymerase, RNA, and buffer were preincubated for 15 min prior to the addition of compound 4.

Surface Plasmon Resonance—Surface plasmon resonance (SPR) analyses were performed using a BIAcore 3000 equipped with a streptavidin sensor chip in a running buffer of 0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% (v/v) surfactant P20, and 0.1% Me2SO. A biotinylated 36-mer RNA (5'-AUAUUAUCCUAUAUACCGGCUUG-3', 2 fold of compound 4 (10 µM) was immobilized to a surface density of 30 response units (RU) by injecting 9 µl of a 25 µM solution at a flow rate of 10 µl/min. The following injection solutions were prepared in running buffer: 10 nM GTP, 100 nM ATP, 100 nM UTP, 250 nM [α-32P]CTP (400 Ci/mmol), 10 mM UTP, Amersham Biosciences), and ~0.2 µl of 10 mM NaCl (1 µM HCV RdRp Inhibitors). Preincubation of the reaction mixture with 10 nM HCV NS5B, and 10 nM compound 4 was utilized as the control reaction. The x axis displays 3-fold dilutions of compound (in nM) and the y axis total cpm of newly synthesized [32P]GTP-labeled RNA product. Data suggest that the benzothiadiazine may interfere with initiation, rather than elongative synthesis. Furthermore, similar results were obtained when using a 3-fold molar excess of poly(A) RNA to trap free polymerase in the poly(rC)-oligo(rG) RdRp assay (data not shown). In these assays, compound 4 activity was similar to that of compound 4 (data not shown).

**RESULTS**

**Single Cycle Synthesis**

The benzo-1,2,4-thiadiazines (compounds 1 and 4) were shown to be potent inhibitors of the HCV RdRp with biochemical IC50 values between 80 and 100 nM and cell-based IC50 values around 500 nM (1). Calorimetry studies confirmed a direct interaction of these compounds with the viral polymerase and a window of selectivity for nucleic acid interaction. The standard RdRp assay described previously (25) represents a continuous polymerization assay rather than single cycle synthesis. The RdRp assay was modified by adding heparin 15 min before initiation of RNA synthesis in an attempt to reduce the number of newly initiated rounds of RNA synthesis. Heparin functions as a nucleic acid mimic to titrate free enzyme, including polymerase molecules, which dissociate from the RNA during elongation. Therefore, in the presence of heparin, new reinitiation events should be impeded, and only RNA synthesized from already initiated complexes or complexes in the elongation phase should be measured.

Using this assay format, compound 4 (1) and a 3'-rdGTP chain terminator were tested. The 3'-rdGTP is incorporated into the newly synthesized RNA product. However, unlike GTP, this analog cannot be extended further because a 3'-hydroxyl group is absent. This chain terminator would be expected to have a similar potency profile regardless of whether it is tested in the presence or absence of heparin, because heparin should not interfere with elongative synthesis. As shown in Fig. 1, A and B, the IC50 for 3'-rdGTP remained at ~100 nM, both in the presence and absence of heparin. As expected, the total amount of radiolabel incorporated into the newly synthesized RNA template decreased around 7-fold in the presence of heparin, an observation consistent with single cycle synthesis. Interestingly, although compound 4 is a highly potent inhibitor in the standard assay (Fig. 1C), it failed to impair the amount of label accumulated in the presence of heparin (Fig. 1D). These data suggest that the benzothiadiazine may interfere with initiation, rather than elongative synthesis. Furthermore, similar results were obtained when using a 3-fold molar excess of poly(A) RNA to trap free polymerase in the poly(rC)-oligo(rG) RdRp assay (data not shown). In these assays, compound 1 activity was similar to that of compound 4 (data not shown).

**Initiation of RNA Synthesis**

To extend the above described study, a more direct assay was developed to examine initiation of RNA synthesis. Recently, Bressanelli et al. (21) identified three NTP-binding sites in the HCV RdRp: a low affinity site specific for GTP and the two nucleotide-binding sites in the active site of the RdRp (NTP1 and NTP2). To discern the mode of action for compound 4, RNA synthesis on a 17-mer RNA (5'-GGCAGCAGCCAGCUAAC-3') was primed using a dinucleotide 5'-GpU3' as the initiation nucleotide (NTP1). NTP1 provides the 3'-hydroxyl for nucleoti-
dyl transfer to a radiolabeled NTP (NTP2; [α-32P]UTP) as shown in Fig. 2A. In the presence of both NTP1 and NTP2, a radiolabeled 3-mer RNA product is present (Fig. 2B, lanes 4 and 5). This 3-mer RNA is a functional substrate for additional nucleotide incorporation, with elongative synthesis products forming after the addition of ATP (Fig. 2B, lane 3), ATP and GTP (lane 2) or all four nucleotides (lane 1). The addition of compound 4 was shown to abrogate the initial phosphodiester bond formation between 5'-GpU3- and radiolabeled UTP (Fig. 2C, compare lane 4 with lanes 5 and 6), regardless of whether ATP and CTP were present (Fig. 2C, lane 1). Furthermore, compound 4 also inhibits the addition of a second nucleotide (ATP) onto the GUU-primed RNA (Fig. 2D, lanes 1–4). Taken together, these data suggest that the thiadiazines interfere directly with the process of RNA initiation and prevent initial

**Fig. 2. Initial phosphodiester bond formation from primer-dependent RNA synthesis.** RdRp reactions were initiated using GpU and 32P-labeled UTP, and in some instances additional cold nucleotides were added to allow formation of elongative products. Reactions were incubated for 20 min prior to analysis on a 25% denaturing gel. A, schematic representation of the NTP1 and NTP2 binding sites within the viral polymerase; the initiation nucleotides and template RNA are shown. B, formation of initiation products in NS5B primer-dependent RNA synthesis (lane 4). Single phosphodiester bond formation was evident upon the addition of ATP (lane 3) but not in the presence of CTP (lane 5). The addition of other nucleotides allowed the extension of initiation product (GpUpU; 5-mer) into slower migrating bands (lanes 1 and 2). C, abrogation of initial phosphodiester bond formation in the presence of benzothiadiazine compound 4 (lanes 1 and 4). The presence of a radiolabeled RNA product indicates that an initial phosphodiester bond was formed and that initiation of RNA synthesis occurred (lanes 2 and 3 and 5 and 6). D, abrogation of second phosphodiester bond formation in the presence of benzothiadiazine compound 4 (lanes 1–4).
were 101, 102, and 110 nM, respectively (Fig. 4). Hence, com-
Kd the RdRp buffer showed an apparent
apoenzyme or appear to disrupt the polymerase-RNA complex.

Compound 4

Three separate reactions were preincubated in the presence of buffer
prior to the initiation of the assay (see symbol legend in inset): Δ21 HCV
RdRp preincubated with RNA, Δ21 HCV RdRp preincubated with com-
pound 4, or compound 4 preincubated with RNA for 15 min. The x axis
displays x-fold dilutions of compound in nM and the y axis total cpm of
newly synthesized [33P]rGTP-labeled RNA product.

De Novo and Primer-dependent RNA Synthesis

Additional studies were performed using RNA LE21 (Fig. 3A), to allow direct and quantitative measurement of both de
novo and primer-dependent RNA synthesis within a single
reaction. GTP is required for de novo initiation of LE21 RNA, and the resultant product is 21 nt (Fig. 3B, lane 1). Two mol-
ecules of LE21 can form a heteroduplex (Fig. 3A) to allow primer extension, which can occur in the absence of GTP, to
generate a 34-nt product (Fig. 3B, lanes 1 and 2) as reported by
Ranjith-Kumar et al. (26). Multimers of 21-nt product (i.e. 42,
63, etc.) are usually observed; this is because of the end-to-end
template switch (recombination) (27). Reactions without com-
pound 4 received a mock aliquot of Me2SO to normalize for any
effects of Me2SO on RNA synthesis (Fig. 3B, lanes 1 and 2).
Upon the addition of 25 nM compound 4 to the reaction (Fig. 3B,
lanes 1–6), both de novo and primer-dependent RNA synthesis
were inhibited by ~39% (Fig. 3C). Significantly less inhibition
was observed at a lower concentration of the heterocyclic
inhibitors.

Nucleic Acid-Polymerase-Compound Interaction

As shown above, the benzothiadiazines disrupted the initia-
tion step of RNA synthesis. It is unclear from these studies
whether the heterocyclic inhibitors form a ternary complex
with polymerase-bound RNA or interact preferentially with the
RdRp apoenzyme prior to template binding. To address this
question, (a) an RdRp assay varying the order of reagent addi-
tion, (b) filter binding studies, and (c) surface plasmon reso-
nance studies were performed.

Order of Addition—The order of reagent addition was varied
for the standard RdRp assay. Specifically, free polymerase was
preincubated with RNA prior to the addition of compound 4,
was preincubated with compound 4 prior to the addition of
template RNA, or was used to initiate reaction for a mixture
containing preincubated RNA and compound. The order of
addition variation did not affect compound potency; IC50 values
were 101, 102, and 110 nM, respectively (Fig. 4). Hence, com-
pound 4 did not demonstrate preferential inhibition for the
apoenzyme or appear to disrupt the polymerase-RNA complex.

Filter Binding—Titration of polymerase with SLD8 RNA in
the RdRp buffer showed an apparent Kd of 62 nM (Fig. 5A).
Titration of compound 4 with the polymerase-RNA complex did
not result in a measurable IC50, suggesting that under these
conditions the heterocyclic inhibitor did not interfere with the
polymerase-RNA interaction (Fig. 5B). This result was consist-
ent with the order of addition experiment shown in Fig. 4.

Compound 4

FIG. 5. Filter binding analysis. A. In vitro binding reactions were
carried out using 5 nM purified Δ21 HCV RdRp in RdRp buffer. Reaction
mixtures containing increasing amounts of [33P] end-labeled SLD8 RNA
were incubated for 15 min at 30 °C and then filtered immediately onto
an 0.45 μm MF-membrane filter. Data are presented to allow calcula-
tion of Kd. The x axis displays dilutions of RNA (in μM) and the y axis
the total cpm of [33P]-labeled RNA retained on the filter. B. For
the binding reaction in the presence of the heterocyclic inhibitor, compound
4 was preincubated with Δ21 HCV RdRp in RdRp buffer for 15 min
prior to the addition of RNA. The x axis displays dilutions of compound
(in μM) and the y axis the total cpm of [33P]-labeled RNA retained on the
filter.
of PFA to inhibit the viral RdRp in primer extension assays (data not shown). Conversely, the response differential for the RNA-bound HCV RdRp upon adding compound 4 or GTP increased to around 27 RU. This change in RU corresponds to an increase in mass on the chip, potentially indicating an increase in affinity for RNA by HCV RdRp bound to compound 4 or GTP. Taken together, these studies confirm that both GTP and compound 4 are capable of interacting directly with the RNA-bound viral RdRp.

**Terminal Transferase Activity—**Recombinant HCV RdRp was reported to possess the ability to add nontemplated nucleotides to the 3’ end of viral RNA, referred to as terminal transferase (TNTase) activity (9, 25). Further, this RdRp-associated activity has been implicated in maintaining the integrity of the termini of the viral RNA genome (25). Because compound 4 was shown not to interfere with the formation of an enzyme-RNA complex necessary for initiation of RNA synthesis, it was evaluated for inhibition of other RdRp functions such as TNTase activity. Compound 4 was found to inhibit TNTase activity of the HCV RdRp with a potency profile equivalent to inhibition of RdRp activity (data not shown).

**DISCUSSION**

The HCV RNA-dependent RNA polymerase, the central catalytic enzyme of replication, represents a viable target for identification of antiviral agents to treat chronic HCV infections. In this study, we report the mechanism of action for the benzothiadiazine compound 4. We describe an assay using a short RNA template to investigate the early steps of HCV RdRp-catalyzed RNA synthesis. Specifically, we have demonstrated that this HCV-selective inhibitor interferes with the initiation process of viral replication rather than with elongative RNA synthesis. The inhibition of initial phosphodiester bond formation occurs regardless of whether replication initiates via a primer-dependent or a de novo mechanism.

The distribution of products suggests that the RdRp initiation complex readily accumulates dinucleotides but may be inefficient in forming a productive elongative complex. This abundance of di- or trinucleotides in the gel-based assays reported herein as well as by another group (28) suggest that the RdRp may dissociate rapidly from the RNA template rather than transition efficiently into elongative synthesis. This process of abortive initiation is not uncommon and has been described recently for T7 RNA polymerase (29–34), such that initiation complexes release short RNA templates prior to transition toward forming a stable elongative complex. This suggests that a conformational change may be inherent in this transition and that HCV may operate in a manner similar to T7 RNA polymerase.

As a first attempt at understanding the dynamic process of RNA synthesis and utilizing compound 4 as a tool, several key experiments were performed. The goal was to assess whether compound 4 inhibits the apoenzyme form of the viral RdRp or is capable of forming a ternary complex to mediate inhibition of RNA synthesis. The order of reagent addition studies showed no difference in the inhibition profile when preincubating the RdRp with RNA or with compound prior to initiating the reaction. These data suggest that a ternary complex may indeed form, although alternate possibilities for the mode of action cannot be directly ruled out on the basis of this data. Similar results were found using the filter binding study, suggesting that an increasing concentration of compound 4 did not disrupt the ability of the RdRp to interact with RNA template. Finally, to evaluate formation of a ternary complex more directly, surface plasmon resonance was utilized. This study provided evidence that a ternary complex is capable of forming between RNA template, Δ21 HCV RdRp, and compound 4. Together, these data suggest that this heterocyclic agent interacts with both the apoenzyme form and the RNA-bound form of Δ21 HCV RdRp and does not interfere directly with RdRp-RNA interaction. The ability to interact with Δ21 HCV RdRp that is tethered to an RNA template is consistent with its inhibition in the replicon system, whereby the HCV RdRp is presumed to be membrane-bound and continually in contact with the viral RNA. Further studies using the full-length form of the viral polymerase will help provide additional insight into this inhibition profile.

The TNTase activity of HCV RdRp, which is responsible for adding nucleotides to the 3’ terminus of template RNA, may directly modulate the initiation of RNA synthesis. This activity potentially could be used by RNA viruses to restore the 3’-initiation site, which may be damaged by exonucleases present in the intracellular compartments (35). Herein, we report that compound 4 disrupts both RdRp and TNTase activity of the HCV RdRp. To date, all evidence indicates that TNTase activity is a property of the viral polymerase that requires the RdRp catalytic site, and inhibition of both enzymatic activities by compound 4 is in agreement with this model.

This report represents the first demonstration of an initiation inhibitor for HCV RNA synthesis. This novel mode of action would be expected to demonstrate synergy with nucleoside analogs capable of inhibiting the HCV RdRp, with the latter class of agents representing elongation inhibitors. Further investigations are ongoing to assess the potential utility of this agent in the treatment of chronic HCV disease.

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