Identification and Biological Characterization of Heterocyclic Inhibitors of the Hepatitis C Virus RNA-dependent RNA Polymerase*

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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. We established a biochemical RNA synthesis assay, using purified recombinant NS5B lacking the C-terminal 21 amino acid residues, to identify potential polymerase inhibitors from a high throughput screen of the Glaxo-SmithKline proprietary compound collection. The benzo-1,2,4-thiadiazine compound 1 was found to be a potent, highly specific inhibitor of NS5B. This agent interacts directly with the viral polymerase and inhibits RNA synthesis in a manner noncompetitive with respect to GTP. Furthermore, in the absence of an in vitro-reconstituted HCV replicase assay employing viral and host proteins, the ability of compound 1 to inhibit NS5B-directed viral RNA replication was determined using the Huh7 cell-based HCV replicon system. Compound 1 reduced viral RNA in replicon cells with an IC_{50} of ~0.5 μM, suggesting that the inhibitor was able to access the perinuclear membrane and inhibit the polymerase activity in the context of a replicase complex. Preliminary structure-activity studies on compound 1 led to the identification of a modified inhibitor, compound 4, showing an improvement in both biochemical and cell-based potency. Lastly, data are presented suggesting that these compounds interfere with the formation of negative and positive strand progeny RNA by a similar mode of action. Investigations are ongoing to assess the potential utility of such agents in the treatment of chronic HCV disease.

Hepatitis C virus (HCV),¹ a positive strand RNA virus of the Flaviviridae family, is the major etiological agent of post-transfusion and sporadic non-A, non-B hepatitis (1). An estimated 2–3% of the world population is chronically infected with HCV, which causes significant liver disease, cirrhosis, and can eventually lead to the development of hepatocellular carcinoma. In infected cells, translation of the viral RNA yields a 3011-residue polyprotein chain (2–4), which is subsequently cleaved to generate envelope and core proteins, for assembly of new virus particles and nonstructural enzymes essential for viral replication (5–7). Studies using recombinant NS5B polymerase have provided direct evidence for RNA-dependent RNA polymerase activity (8, 9), and this catalytic activity has been confirmed to be required for infectivity in chimpanzees (10). NS5B polymerase contains a hydrophobic C-terminal domain thought to be responsible for anchoring the protein to mammalian cell membranes. Removal of the C-terminal 21 residues has been reported to facilitate protein isolation from Escherichia coli without compromising RdRp activity (11). The HCV RdRp initiates RNA synthesis preferentially from the 3’ terminus of the template RNA (12, 13–15) but lacks specificity for HCV RNA in vitro, because it readily utilizes heterologous nonviral templates (8). Based on crystallographic studies of the enzyme containing C-terminal truncations (16, 17), the hydrophobic tail present in the full-length enzyme could be predicted to partially occupy the palm domain, and hence may interfere with identification of active site inhibitors in biochemical assays. Although modeling experiments suggest that a template-primer can be readily accommodated into the truncated polymerase without altering the global folding, it is unclear whether this model can be extrapolated to the full-length RdRp that exists within the functional replicase complex.

A detailed understanding of the mechanisms regulating HCV replication has been plagued by the lack of an efficient virus culture system. Recently, however, cell-based replicon systems for HCV were developed in which the nonstructural proteins stably replicate subgenomic viral RNA in Huh7 cells (18, 19). 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 development of a robust RdRp

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¹ The abbreviations used are: HCV, hepatitis C virus; RdRp, RNA-dependent RNA polymerase; DTT, dithiothreitol; pol, polymerase; RT, reverse transcription; CD, circular dichroism; NTR, non-translated region; NCR, non-coding region; XTT, (2,3-bis[2-methoxy-4-nitro-5-sulfo-phenyl]-2H-tetrazolium-5-carboxanilide salt); BVDV, bovine viral diarrhea virus; GBV-B, GB virus-B.

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HCV RNA Polymerase Inhibitors

Cytotoxicity Assays—XTT cytotoxicity assays were performed in HuH7 cells. Briefly, 8000 cells/well were cultured overnight in 96-well plates at 37°C and 5% CO2 with Dulbecco’s modified essential medium containing 10% fetal calf serum and 1% nonessential amino acids. Test compounds (10 μM stocks) were diluted serially 1:2 in Me2SO, and 100 μl was added to each well. Following incubation for 4 days, the plates were incubated for an additional 40 h, followed by addition of 50 μl of XTT/phenazine methosulfate (Sigma) solution to a final concentration of 400 μg of XTT and 3 ng of phenazine methosulfate per well. Plates were further incubated up to 4 h until the A492 value was between 1.5 and 1.8 to allow IC50 calculation.

Positive Strand Replicon RNA Detection—Replicon cells were plated at 1000 cells per well in 96-well plates in 100 μl of Opti-MEM (Life Technologies). A 10-μl aliquot of Fugene HD (Promega) was applied to Talon metal affinity resin (CLONTECH), and the bound proteins were step-eluted with 30 and 200 mM imidazole. The 200 mM eluate was dialyzed against buffer A containing 20 mM Tris-Cl, pH 8.0, 3 mM DTT, 150 mM NaCl, and 10% glycerol and applied to poly(U)-Sepharose 4B (Amersham Biosciences) column. After washing with 10 column volumes with buffer A, the protein bound was eluted with buffer A plus 1 mM NaCl. NSSB containing fractions were pooled and concentrated to 30 mg/ml using a centricron 10 concentrator (Amicon). Purity of NSSB as judged by SDS-PAGE was >95%.

Biochemical RdRp Assays—The high throughput RdRp assay was carried out in 384-well plates using 50 mM enzyme, 0.2 μM of [α-32P]GTP, 200 μl of purified NSSB, 20 μM Tris-Cl, pH 7.5, 5 mM MgCl2, 25 mM KCl, 3 mM DTT, and 0.05% bovine serum albumin. The 25-μl reaction was terminated after 2 h at 25°C upon addition of equal volume of 100 mM EDTA and transferred to a streptavidin-coated FlashPlate. After incubation at 25°C for 30 min, and the plate was washed extensively and counted using a Packard TopCount microplate reader (n = 4 for secondary screening). Additionally, viral RdRps (20, 21) and DNA polymerase were tested using 1 M Tris, pH 9.0.

Negative Strand Replicon RNA Detection—To achieve strand-specific detection, a primer containing HCV RNA sequences and an 18-base tag located at a position of interest was synthesized (RT) reaction, 5′-ACATCGGCGCATGAGGACCGCGCATGACGTTAG-3′. A Thermoscript-RT-PCR system (Invitrogen) was used for the RT reaction according to the manufacturer’s protocol, with ~9 μl of the cell-harvested RNA and 1 μl of primer (10 μM) incubated with RT at 60°C for 1 h. Following that incubation, 2 μl of cDNA product containing the 5′ tag was amplified for TaqMan quantification using the 48 μl of TaqMan Universal Master Mix (Applied Biosystems) as well as primers: neo-forward, 5′-CCGCGCTTTGACGCGCGAAG-3′; neo-reverse, 5′-CCGCGCTTTGACGCGCGAAG-3′; neo probe, 5′-FAM-ACATCGGCGCATGAGGACCGCGCATGACGTTAGCAGGAGCAGCAGGACACGTGCACCCATGACGTTAG-3′. Samples were mixed briefly and placed in an ABI 7500 (Applied Biosystems) at 50 °C; 2 min; 60 °C, 30 min; and 95 °C, 5 min; with cycling parameters set to 94 °C, 20 s; 55 °C, 1 min for 40 cycles. Phosphorimaging. 

Circular Dichroism (CD) Spectroscopy—CD analysis was performed on purified HCV Δ21 NSSB (0.3 mg/ml in 25 mM Tris, pH 7.5, 25 mM KCl, 5 mM MgCl2, and 0.8% Me2SO) at 20°C in the presence (40 μM) and absence of the inhibitors. Far-UV CD spectra were recorded on a Jobin-Yvon CD spectrometer with a water-jacketed cuvette holder with path length. Data (10 accumulations) were collected using a time constant of 2 s with a 1-nm constant spectral bandwidth at 50 nm/min (CD wavelength scans not shown). Thermal stability of HCV Δ21 NSSB was measured by scanning temperature at 1°C/minute while monitoring the CD signal at 220 nm. Thermal stability data were then analyzed using GraFit and a fitting equation adapted from Ref. 22 to predict the midpoint transition temperature (Tm).
Nucleic Acid Binding—Poly(rC) (294 μM bases, Amersham Biosciences) and biotinylated rG13 (208 μM bases) or poly(rA):poly(rU) were added to 1× buffer consisting of prefiltered 10 mM Tris-HCl, pH 7.5, 0.5 mM NaCl, 5 mM EDTA, 1× RNA-Secure (Ambion). The sample was denatured at 60 °C for 15 min, 95 °C for 3 min, and annealed at 37 °C for 30 min. Fluorescence displacement curves were determined by titrating 66 nM PicoGreen (Molecular Probes) and double-stranded RNA (poly(r13G):poly(rC) (112 nM or 1.4 μM bases) or poly(rA):poly(rU) (100 nM)) with 0–100 μM compound in 20 mM Hepes or Tris buffer, pH 7.4, 25 mM KCl, 7.5 mM MgCl2, 3 mM DTT, 0.3 mM NaCl containing 1% MeSO in a continually stirred cuvette maintained at 20 °C. Excitation fluorescence of the PicoGreen-RNA complex at 480 nm and emission at 520 nm were measured using an SLM-8000 spectrofluorometer. Fluorescence of the PicoGreen-RNA complex at 480 nm and emission at 520 nm were measured using an SLM-8000 spectrofluorometer. Fluorescence was monitored in the absence and presence of compound or RNA.

Biophysical Characterization of Benzo-1,2,4-thiadiazines—Mechanistic studies regarding enzyme selectivity and mode of action were performed to characterize the differential inhibition profile of derivatives 1–4 for the HCV RdRp.

Selectivity—Similar IC50 values for compound 1 and 4 were obtained when the RNA substrate for the viral RdRp was changed to oligo(U)-primed homopolymeric poly(rA), or unprimed heteropolymeric RNA (data not shown), indicating the absence of substrate-specific inhibition. Compounds 1–4 were tested for selectivity against closely related viral RdRps (BVDV and GBV-B) and mammalian polymerases (DNA pol α and DNA pol B), and all IC50 values were >50 μM with a selectivity index above 500.

Polymerase Interaction—CD spectroscopy was used to monitor thermal denaturation of NS5B in the presence of either MeSO or 40 μM inhibitor. Notably, the polymerase-compound 1 complex melted over a broad temperature range (Tm) resulting in a 3 °C increase (Table II), whereas compound 3 resulted in a 0.7 °C increase. Slope kinetics for compound 1-polymerase complex unfolding under these experimental conditions could explain this delayed transition. The polymerase-compound 4 complex resulted in a similar shift in Tm (∆Tm = 3.4 °C), although with a lower magnitude than compound 1 (Fig. 1). Additionally, the affinity of these derivatives for the viral RdRp remained unchanged in the presence of poly(rC) 25-mer or a 3′-ribodeoxy GTP chain terminator (data not shown).
Studies using analytical ultracentrifugation and isothermal titration microcalorimetry further confirmed these observations (data not shown).

**Nucleic Acid Interaction**—Aminoquinoline agents have been shown previously to interact with nucleic acid (24), and a similar compound known to bind to both single-strand and double-strand nucleic acid was used as a positive control. In the PicoGreen displacement assay, this aminoquinoline had a C50 value of 0.03 μM (Fig. 2). The active inhibitors had C50 values for double-strand RNA of about 50 and 0.5 μM, respectively, indicating that the reduction in polymerase activity most likely did not occur by titration or binding of the RNA substrate. The selectivity index comparing nucleic acid interaction with biochemical potency was 500 for inhibitor 1, and 6 for inhibitor 4.

Solubility limitations precluded testing of inhibitor 4 at higher concentrations in this RNA displacement assay. Compounds 2 and 3 had C50 values > 50 μM (data not shown). Consistent with these observations, similar results were obtained in the presence and absence of 1 mM magnesium ions or when using alternative nucleic acid substrates such as poly(A)-poly(U) RNA or with double-stranded DNA (data not shown).

**Noncompetitive with GTP**—Consistent with a previous report (25), the Km for GTP using the Δ21 NS5B preparation on a poly(rC):oligo(rG) RNA substrate was shown to be 0.73 μM (data not shown). Furthermore, mechanistic enzymology studies with compound 1 suggest that this inhibitor exhibits a kinetic behavior consistent with a reversible, noncompetitive mechanism of inhibition with respect to GTP. Consistent with this observation, compound 1 was unable to block the binding of GTP to the viral polymerase. In this study, the Km for GTP remained unchanged while the Vmax decreased with increasing concentration of compound 1 (Fig. 3A). However, as expected, the Km for GTP significantly increased upon titration of 3'-ribodeoxy GTP while the Vmax remained unchanged (Fig. 3B).
Therefore, inhibition by 3'-ribodeoxy GTP was competitive with respect to GTP, and the Lineweaver-Burk plots indicated that compound 1 most likely interacts with the viral polymerase at a site distinct from the GTP binding site. Consistent with these data, compound 4 was also shown to be noncompetitive with GTP (data not shown).

**Biological Validation of Benzo-1,2,4-thiadiazines as Inhibitors of HCV Replication**—The membrane permeability profile for the benzo-1,2,4-thiadiazine compounds 1–4 suggested high cellular permeability (e.g. for compound 1) the rate of permeability through an artificial cholesterol/phospholipid membrane was ~700 nms−1). XTT cytotoxicity assays were performed in both Huh7 replicon cells and parental replicon-naive Huh7 cells. Consistent with the membrane permeability data, XTT CC50 values ranged between 40 and 75 μM for the benzo-1,2,4-thiadiazine compounds 1–4, suggesting cellular penetration.

For viral reduction assays, TaqMan was utilized to monitor both cellular and viral RNA. Cyclophilin RNA levels were normalized to positive-strand HCV viral RNA to allow an accurate IC50 determination upon compound titration, and no changes in the level of cellular RNA were evident upon addition of up to 10 μM of compound 1, further confirming the lack of cytotoxicity in these cells. Cell-based inhibition of viral replication was confirmed with compound 1 in the HCV replicon system, with an IC50 of 0.55 μM (Table III, n = 8) and a therapeutic index relative to cytotoxicity of ~100. Expectedly, benzo-1,2,4-thiadiazines 2 and 3 did not exhibit the ability to inhibit viral replication in the replicon system, when tested at concentrations at or below 20 μM (data not shown). Consistent with the similarity in biochemical potency between compounds 1 and 4, compound 4 showed activity in the replicon system with an IC50 of 0.52 μM for reduction in positive strand viral RNA. Percent reduction in viral RNA was 80% for compound 1 and 91% for compound 4 at 10 μM.

**Impact of Benzo-1,2,4-thiadiazines on Replication Intermediates**—Positive strand viral RNA represents the nucleic acid strand that is translated and initially copied to generate the negative strand replicative intermediate. Negative strand RNA is the template used to generate the positive strand message, which is generally packaged into productive virions. Although the replicon system does not generate infectious particles, because the coding elements for the structural polyproteins have been removed from the replicon construct, monitoring a reduction in positive strand RNA represents a facile method for quantifying activity of polymerase inhibitors. However, the formation of positive and negative strand RNAs would not necessarily require identical replicase conformations, mechanistic requirements, or similar sets of cofactors. To perform mode of action studies and potentially differentiate inhibitors, it is useful to confirm whether antiviral agents inhibit both positive and negative strand RNA synthesis. To that end, the activity profile for compounds 1 and 4 was assessed in a biochemical gel-based RdRp assay using the native termini of the viral genome (3'-NTR RNA and the negative strand of 5'-NTR RNA) as well as in the cell-based replicon by monitoring the reduction in negative strand replicon RNA.

The inhibition profile for compounds 1 and 4 in an RdRp assay using the native HCV termini as RNA substrate, showed a 3-fold increase in potency for compound 4 relative to compound 1 (Fig. 4), compared with less than a one-fold difference in IC50 between these compounds when using homopolymeric RNA. Furthermore, since the IC50 values for a single compound were similar for both positive or negative strand RNAs (5’ RNA IC50 = 0.17 μM for 1 and 0.06 μM for 4; 3’ RNA IC50 = 0.14 μM for 1 and 0.04 μM for 4), this suggested that the mechanism of inhibition for positive strand RNA synthesis is likely to be similar to that for inhibition of negative strand RNA formation for these compounds. The inhibition profile from compounds 2 and 3 in this gel-based assay was similar to that reported for the primer-extension assay detailed in Table I (e.g. less than 30% inhibition at 50 μM). Interestingly, the Δ21 HCV was less efficient at generating the full-size 600-base product from the 5'-NTR negative strand RNA, as shown by the presence of smaller sized termination products (~400–500 bases), compared with products from the 3'-NTR substrate (Fig. 4).

To evaluate whether this shared similarity in strand-specific inhibition occurs in the replicon cells, this system was first validated as a surrogate model for viral infection by confirming the presence of a disparate ratio of positive to negative strand RNA. Levels of positive strand RNA have been reported to be at least 10-fold higher than negative strand in infected liver tissues (26). In the replicon cells, TaqMan analysis showed that ~2200 positive strand replicon RNA copies per cell were present, whereas 200 copies per cell of negative strand were detected (data not shown). Using this assay, compounds 1 and 4 showed a similar inhibition profile for reductions in either

| Table III | Biological inhibition profile of benzo-1,2,4-thiadiazines in replicon cells |
| --- | --- | --- |
| | Positive strand | Negative strand |
| Derivative 1 | 0.55 ± 0.12 | 0.40 ± 0.17 |
| Derivative 4 | 0.52 ± 0.11 | 0.53 ± 0.17 |

**Fig. 4. RdRp using HCV 5'- and 3'-NTR substrates.** RdRp reactions were performed with negative strand RNA from the 5'-NTR (A and C) or the positive strand RNA from the 3'-NTR (B and D). Compounds were titrated using 3-fold dilutions up to 200 μM for compound 1 (A and B) or up to 133 μM for compound 4 (C and D). α-32P-Labeled RNA products were electrophoresed on an 8% TBE gel, and the phosphorimaging analyses were quantified using ImageQuaNT analysis.
positive or negative strand RNA (Table III), with IC_{50} values ranging from 0.4 to 0.55 μM. These data are consistent with the suggestion that these inhibitors exert an equivalent effect on replication of both positive and negative strand RNA synthesis.

**DISCUSSION**

The HCV RNA-dependent RNA polymerase, a 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 development of a practical RdRp assay suitable for high throughput screening. We have demonstrated herein that screening of panels of drug-like molecules with soluble recombinant HCV NS5B in a ribonucleotide incorporation assay resulted in the identification of inhibitors of viral RNA synthesis. Furthermore, the benzo-1,2,4-thiadiazine derivatives were shown to be highly selective for the HCV RNA polymerase, failing to inhibit other viral and mammalian polymerases. Significantly, these compounds retained absolute selectivity for HCV, because they were completely inactive at inhibiting GBV-B virus NS5B RNA synthesis even though the two enzymes share 37% identity and 52% similarity.

Mechanistic studies confirmed that the reduction in viral RNA synthesis by the benzo-1,2,4-thiadiazines was most likely due to direct catalytic inhibition, rather than titration of nucleic acid or competition with nucleotides. Consistent with this observation, compound 1 demonstrated no interaction with nucleic acid at concentrations up to 50 μM. The exact mechanism of inhibition remains unclear. Because compound 4 shares similar structure as well as biochemical and cell-based activity with compound 1, the apparent enhanced interaction with RNA for compound 4 is unclear. The solubility limitations of compound 4 may indirectly affect interpretation of the RNA binding data.

Despite the use of a C-terminally truncated polymerase for high throughput screening, the benzo-1,2,4-thiadiazines were capable of inhibiting polymerization of the full-length NS5B in biochemical assays with a relatively similar potency profile. Most importantly, two of the analogs in the series were able to inhibit the full-length enzyme in the context of the replicase complex expressed in HCV replicon cells. This replication complex most likely consists of cellular and non-structural viral polypeptides. The implication associated with activity in the replicon cells is that the inhibitors were able to affect the perinuclear membrane, the site of viral RNA synthesis. In addition to the reduction in viral RNA, the cytotoxicity and artificial membrane permeability data for these compounds are consistent with cellular penetration. Furthermore, these compounds were unlikely to disrupt replicon RNA synthesis by altering local membrane integrity, because they did not affect virus production in a BVDV plaque assay when tested up to 10 μM, which requires membrane-localized replication for virus propagation (data not shown).

Because the virus generates a negative strand replicative intermediate to use as a template for producing positive strand RNA, we investigated whether these compounds were equally capable of inhibiting both viral replication processes. Individually, compounds 1 and 4 shared similar activities of inhibition for both negative and positive strand RNA replication in the biochemical assay. Furthermore, data from the cell-based replicon system was consistent with these observations and indicate that the mode of action does not appear to be distinct for these two replication processes. Preliminary structure activity relationships on screening hit 1 led to the identification of analog 4 showing similar potency in both biochemical and cell-based assays. Investigations are ongoing to assess the potential utility of such agents in the treatment of chronic HCV disease.

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