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Inhibition of native hepatitis C virus replicase by nucleotide and non-nucleoside inhibitors

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Abstract

A number of nucleotide and non-nucleoside inhibitors of HCV polymerase are currently under investigation as potential antiviral agents to treat HCV-infected patients. HCV polymerase is part of a replicase complex including the polymerase subunit NS5B together with other viral and host proteins and viral RNA. The RNA synthesis activity of the native replicase complex was inhibited by 3'-deoxy-CTP, a chain-terminating nucleotide analog, but not inhibited by non-nucleoside NS5B polymerase inhibitors of three different structural classes. The HCV replicase was also resistant to heparin, a broad-spectrum, RNA-competitive polymerase inhibitor. Prebinding of the recombinant NS5B protein with a RNA template rendered the polymerase largely resistant to the inhibition by heparin and the non-nucleoside inhibitors, but did not affect the inhibitory potency of 3'-deoxy-CTP. Therefore, the HCV replicase showed a similar pattern of inhibitor sensitivity as compared to RNA-bound NS5B. These results suggest that the native HCV replicase complex represents a stable and productive polymerase–RNA complex. The allosteric non-nucleoside NS5B polymerase inhibitors are inactive against established HCV replicase but may function antagonistically with the formation of a productive enzyme–template complex.

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Introduction

RNA replication of hepatitis C virus (HCV) occurs in cytoplasm and is mediated by a membrane-associated replicase complex consisting of many if not all of the NS proteins and possibly host factor(s) (Moradpour et al., 2003). NS5B, the RNA-dependent RNA polymerase (RdRp), is the catalytic subunit of the replicase complex and therefore constitutes a prime target for anti-viral intervention. Using recombinant NS5B protein for screening, both nucleotide and non-nucleoside inhibitors of

HCV polymerase activity have been identified. Nucleotide analogs target the polymerase active site, are mostly competitive inhibitors relative to the natural nucleotide substrates, and can cause chain termination upon incorporation into the RNA molecules (Carroll et al., 2003; Migliaccio et al., 2003; Shim et al., 2003). Non-nucleoside inhibitors of NS5B RNA synthesis activity have been independently identified by several laboratories and belong to a number of different structural classes (Beaulieu et al., 2004; Chan et al., 2004a, 2004b; Dhanak et al., 2002; McKercher et al., 2004). Based on the location of resistance conferring amino acids on NS5B, cross-resistance studies and co-crystal structure information, benzimidazole, benzothiadiazine, and thiophene carboxylic-acid-based compounds bind to three non-overlapping binding sites on NS5B (Love et al., 2003; Nguyen et al., 2003; Tomei et al., 2003, 2004; Wang et

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al., 2003). Benzothiadiazine and benzimidazole inhibitors did not inhibit NS5B activity under single-cycle RNA synthesis conditions (Gu et al., 2003; McKercher et al., 2004). Therefore, the potency of these non-nucleoside inhibitors may be dependent on the status of the enzyme–template interaction, and HCV polymerase inhibition may only be occurring during the initiation phase of RNA synthesis. Despite the widespread use of recombinant NS5B as an *in vitro* model of HCV-specific RNA synthesis, the impact of NS5B interaction with other HCV replicase subunits on the inhibitory potency of polymerase inhibitors remains unclear. The HCV replicase system in hepatoma cells provides a source of native replicase complex, which could be used to address this issue, independent of potential differences in cell penetration or cellular toxicity between investigational compounds. Here, the inhibition of HCV replicase activity was determined using representative examples of non-nucleoside inhibitors of three different structural classes and a nucleotide analog.

Results

Biological characterization of membrane-associated replicase complex

HCV polymerase NS5B is a membrane-associated protein and part of a multiprotein replicase complex (Moradpour et al., 2003). A cell-free preparation of enzymatically active HCV replicase complex was isolated as a cytoplasmic membrane fraction from Huh-7 cells harboring a subgenomic NS3-5B replicon (Krieger et al., 2001) by Dounce homogenizing and differential centrifugation (Takeda et al., 1986). The HCV protein composition of the cytoplasmic replicase complex (CRC) obtained by this method was determined by Western blotting analysis. NS3, NS4B, NS5A, and NS5B were present in the CRC fraction from replicon cells, but not in the corresponding membrane fraction from parent, replicon-free Huh-7 cells (Fig. 1A). As a control, GRP78, a chaperone protein residing in endoplasmic reticulum (ER), was confirmed to

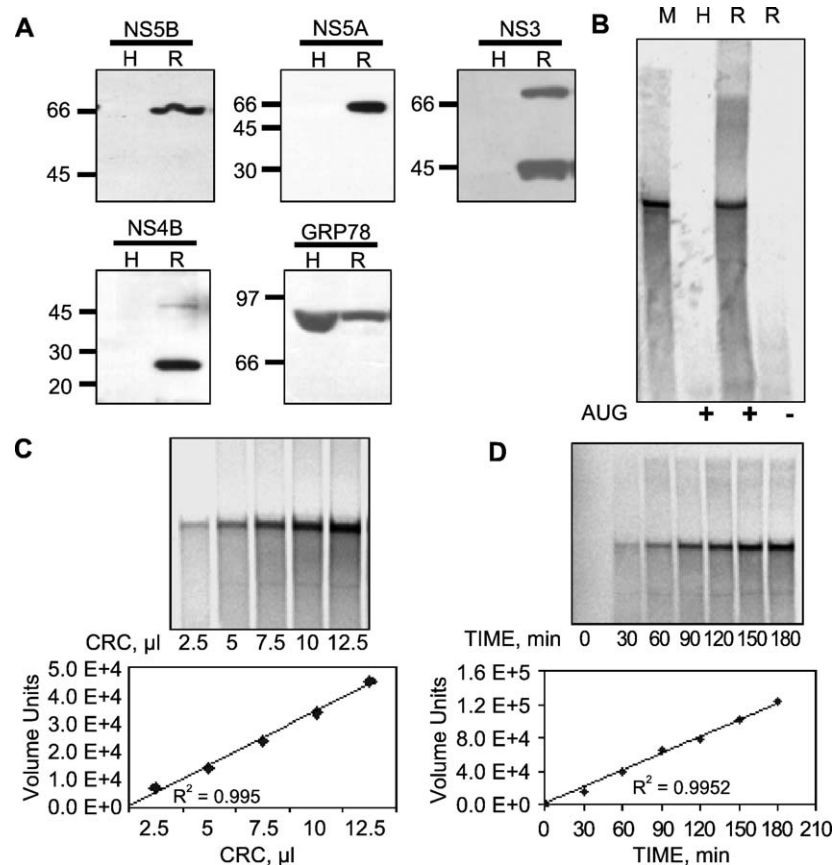


Fig. 1. Isolated CRC contains multiple HCV NS proteins and is enzymatically active. (A) Detection of HCV nonstructural proteins and endoplasmic reticulum-residing protein GRP78 in the membrane fraction from Huh7 (H) or replicon (R) cells. Molecular weights of proteins are indicated on the left. Rabbit polyclonal antisera against NS5B, NS5A, and NS3 were developed using recombinant NS5B, NS5A, or NS3 protease domain as immunogens. Monoclonal antibody against NS4B (4B52) was kindly provided by Dr. Kohara (Tsukiyama-Kohara et al., 2004). (B) NTP-dependent *in vitro* RNA synthesis activity of cytoplasmic membrane fractions. Membrane fraction from Huh7 (H) or replicon (R) cells was incubated with [α - 32 P]-CTP in the presence or absence of 1 mM each of ATP, UTP, and GTP (AUG). M, radiolabeled single-strand replicon RNA. (C) Enzyme-dependent RNA synthesis activity of replicase. (D) Time course of replicase assay in which 10 μ l of CRC was included in the reactions for indicated time points. RNA bands were quantitated and plotted as shown on the lower panel. R^2 values represent the goodness of linear regression of the data.

be present both in the membrane fractions from parent Huh-7 cells as well as Huh-7-derived cells containing HCV replicon (Fig. 1A). In the presence of exogenous nucleotide triphosphates (NTPs), the replicase complex was able to synthesize full-length copies of the endogenous replicon RNA template (Fig. 1B). The major product of RNA synthesis by CRC was full-length replicon RNA. The RNA product was not seen with the corresponding membrane fraction from Huh-7 parent cells or when only a single NTP was provided (Fig. 1B). Therefore, the synthesis of HCV replicon RNA *in vitro* was HCV-replicase-specific and NTP-dependent. The replicase-associated RNA synthesis activity was dependent on the amount of protein complex in the reaction. A linear increase of product formation was observed up to 12.5 μ l CRC, where 1 μ l of CRC represented membrane fraction from 5×10^5 cells (Fig. 1C). In the presence of 10 μ l CRC, full-length RNA synthesis also increased linearly with time up to 180 min (Fig. 1D). Similar results were obtained with multiple CRC preparations suggesting highly robust and reproducible HCV replicase activity obtained by using the current cell fractionation protocol. Ten-microliter CRC and an incubation time of 120 min were established as standard replicase assay conditions for inhibitor characterization. According to comparative Western blot analysis using recombinant NS5B as a reference, the NS5B concentration in the standard replicase assay was similar within 2-fold to that used in the standard recombinant NS5B polymerase assay (data not shown).

Inhibition of replicase activity by nucleotide analog and non-nucleoside inhibitors

A number of HCV polymerase inhibitors have been identified using recombinant NS5B proteins in isolation from other replicase subunits *in vitro*. Different compounds were selected to compare inhibitory potencies using *in vitro* RNA synthesis assays with HCV replicase (CRC) or recombinant HCV polymerase (NS5B570-BK). The inhibitors tested include a chain-terminating nucleotide analog, 3'-deoxycytidine triphosphate (3'-dCTP), and a representative molecule from each of three different classes of non-nucleoside inhibitors: compound 1 based on a benzimidazole scaffold (Beaulieu et al., 2004; Dhanak et al., 2002), compound 2 based on a benzothiadiazine structure (Dhanak et al., 2002), and compound 3 based on a thiophene carboxylic acid scaffold (Chan et al., 2004a, 2004b) (Fig. 2A). Based on structural and mutational analysis, these four compounds bind to distinct binding sites on NS5B. 3'-dCTP significantly inhibited the RNA synthesis activity of the HCV replicase, with complete inhibition achieved at 7.5 μ M (Fig. 2B). Surprisingly, none of the non-nucleoside inhibitors showed an inhibitory effect on HCV replicase activity at concentrations up to 100 μ M, despite full inhibition of replicase by 7.5 μ M 3'-dCTP in control reactions (Fig. 2B). 3'-dCTP inhibited RNA synthesis

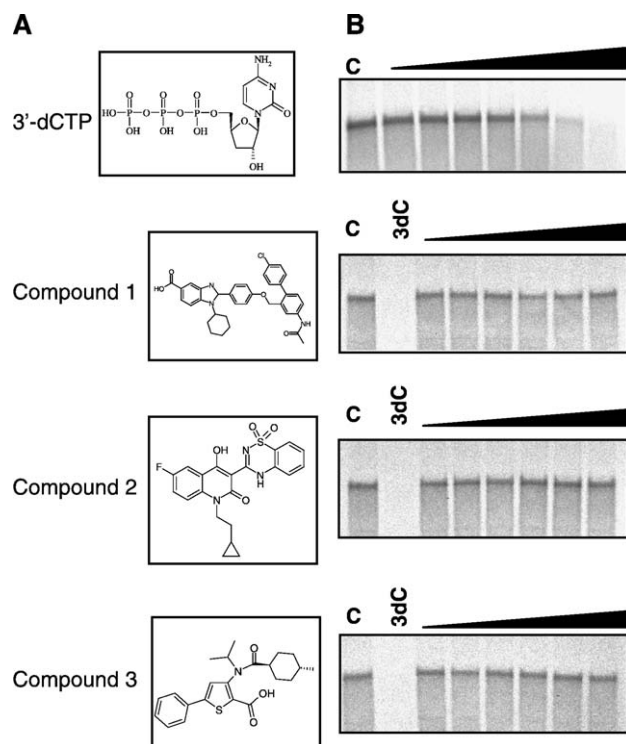


Fig. 2. Effect of nucleotide and non-nucleoside inhibitors on replicase activity. (A) Structures of inhibitor compounds. (B) Inhibition of replicase activity by nucleotide and non-nucleoside inhibitors. Replicase activity was determined in the presence of nucleotide analog 3'-dCTP at concentrations of 0.01, 0.03, 0.09, 0.28, 0.83, 2.5, and 7.5 μ M, or each of the non-nucleoside inhibitors at concentrations of 0.032, 0.16, 0.8, 4, 20, and 100 μ M. C, replicase activity in the absence of inhibitor; 3dC, replicase activity in the presence of 7.5 μ M 3'-dCTP.

activity of recombinant NS5B and the replicase complex with similar potency (Table 1). All three non-nucleoside inhibitors tested inhibited the RNA synthesis activity of NS5B570-BK on a heteropolymeric RNA template derived from the 3'-end of the negative-strand HCV genome (cIRES) with submicromolar IC_{50} values, similar to previously published values (Table 1). The RNA synthesis activity of NS5B was inhibited to more than 90% completion by each of the three non-nucleoside inhibitors at 100 μ M (data not shown), a concentration at which replicase activity was not inhibited.

Replicase activity is resistant to heparin inhibition

The different sensitivity of the recombinant RdRp and the replicase to the inhibition of allosteric non-nucleoside inhibitors suggested that the RdRp in the replicase complex may assume a different conformation due to its interaction with the endogenous HCV RNA template, other viral proteins, or host cofactors. Sensitivity of the RdRp activity to heparin was used to test for differences in RNA turnover between recombinant NS5B and native HCV replicase. Heparin is a competitive inhibitor of nucleic acid binding and can function as a polymerase-trapping agent binding to

Table 1
Inhibition of HCV replicase and recombinant NS5B RNA synthesis activity by nucleotide and non-nucleoside inhibitors

RNA synthesis assay	IC ₅₀ [μM] ^a			
	3'-dCTP	Compound 1	Compound 2	Compound 3
Replicase	0.31 ± 0.18	>100	>100	>100
NS5b570	0.33	0.05 ± 0.01	0.1 ± 0.04	0.29 ± 0.04

^a Values represent means and standard deviations of IC₅₀ values determined from at least three independent experiments.

and inhibiting free polymerase molecules, whereas productive polymerase–template complexes are resistant to inhibition by heparin (Bambara et al., 1995; Tomei et al., 2000). The replicase complex was preincubated with heparin for 10 min before the addition of assay buffer and NTP mixture to allow replicase–heparin equilibration prior to initiation of RNA synthesis. Fig. 3A shows full-length HCV replicon RNA synthesis by CRC in the presence of heparin up to 1 mg/ml. The RdRp activity of the HCV replicase was completely resistant to inhibition by heparin under these conditions, consistent with a highly stable polymerase–template RNA interaction in the CRC. In contrast, the RNA synthesis activity of the recombinant NS5B protein was sensitive to inhibition by heparin and completely inhibited at heparin concentrations above 100 μg/ml (Fig. 3B). These results suggest that the native HCV replicase exists as a stable and productive polymerase–RNA complex. Heparin resistance of the replicase also indicates that the replicase undergoes limited template RNA turnover under the assay conditions, generating multiple copies of product from each template. Alternatively, the RNA product generated by the replicase may represent a single round of RNA replication from the elongation of replication intermediates present at the time of CRC preparation. Several lines of evidence support the first hypothesis. Labeled RNA product generated by CRC increased linearly with time up to 180 min, which is much longer than the estimated time requirement for a single round of full-length replicon RNA synthesis based on the previously reported elongation rate of 700 nucleotides per minute of the HCV polymerase in vitro (Tomei et al., 2000). Full-length RNA product was detectable very early in the replicase reaction and increased in intensity linearly with time, consistent with the continuous synthesis of new full-length copies (Fig. 1D). Studies on the replication rates of other viruses from the *Flaviviridae* family, Kunjin and Dengue virus, showed that the cycle of viral RNA synthesis to complete one full-length strand was about 15 min (Chu and Westaway, 1985), consistent with the detection of full-length transcripts generated by HCV replicase at early reaction times. A recent study on the replicase complex of bovine viral diarrhea virus showed that the BVDV replicase retained a large proportion of RNA synthesis activity even in the presence of 2 mg/ml of heparin (Tomassini et al., 2003). Therefore, stable protein–template RNA interaction may be a conserved feature of native,

membrane-associated replicase complexes among viruses of the *Flaviviridae* family. These results are different from those described by Hardy et al. (2003), which showed high heparin sensitivity of the HCV replicase purified from replicon cells. The reason for the loss of heparin resistance in that membrane preparation is unclear, but may be related to slightly different cell fractionation protocols leading to the loss of a stabilizing cofactor.

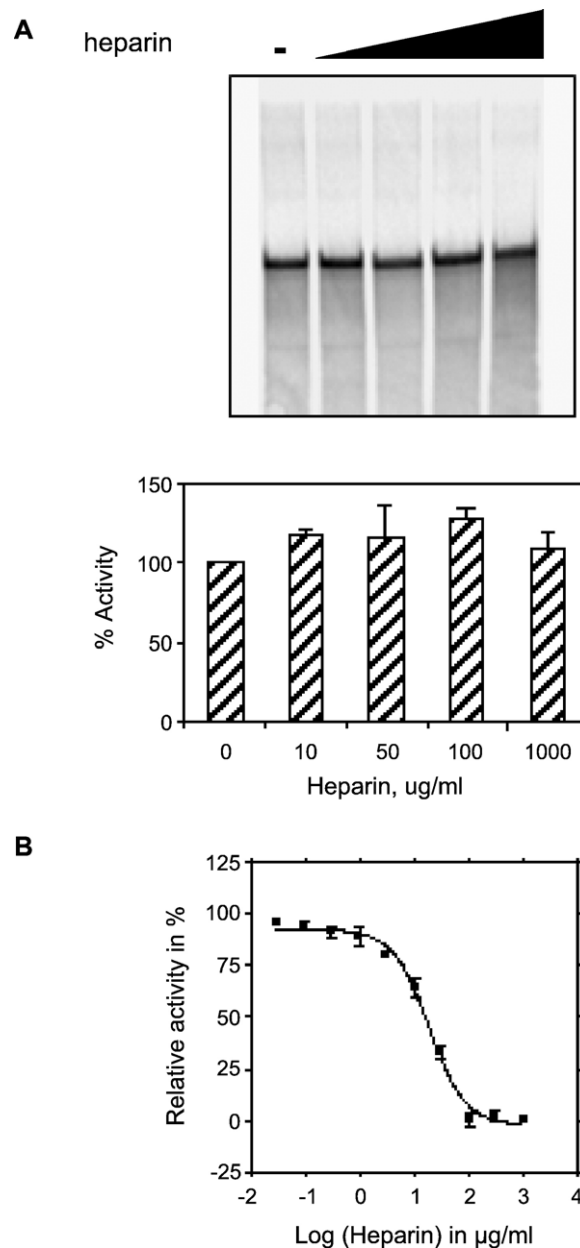


Fig. 3. Differential sensitivity of HCV replicase and NS5B to the inhibition by heparin. (A) Replicase is resistant to heparin inhibition. Replicase activity was determined in the absence (–) or presence of heparin at the indicated concentrations. RNA product in the presence of heparin was expressed as a percentage of the RNA product synthesized in the absence of heparin and plotted. (B) Inhibition of recombinant NS5B by heparin.

Effect of RNA–NS5B complex formation on the inhibition properties of three classes of non-nucleoside compounds

The more stable interaction of polymerase with RNA template in the replicase complex as compared to recombinant NS5B may be related to the resistance of the replicase to inhibition by non-nucleoside compounds. In order to determine whether stable interaction with RNA template was sufficient to generate resistance to inhibition by non-nucleoside inhibitors and heparin, recombinant NS5B was preincubated with RNA templates before assessment of inhibitor potencies. NS5B was preincubated with the heteropolymeric RNA template for a period up to 4 h before the addition of heparin or each of the three non-nucleoside compounds (Fig. 4). NS5B was inhibited to more than 90% completion under these conditions, when heparin or non-nucleoside inhibitors were added together with template RNA (Fig. 4, time 0). As compared to the activity in the absence of inhibitors, the RdRp activity of NS5B became increasingly resistant to inhibition by heparin, when NS5B was prebound to template RNA before the addition of heparin and initiation of RNA synthesis (Fig. 4, left panel). Acquiring resistance to heparin by RNA binding was a slow process, reaching maximal effect at about 2 h of preincubation of NS5B with HCV cIRES RNA, suggesting a slow process of stable RNA complex formation, possibly related to an RNA-induced protein conformational change. Similar to the results obtained with heparin, the RNA synthesis activity of NS5B became largely resistant to inhibition by different structural classes of non-nucleoside compounds after NS5B preincubation with HCV cIRES RNA (Fig. 4, middle and right panels). As observed with heparin, acquiring non-nucleoside resistance by RNA binding was a slow process, reaching plateau levels after 2 h of enzyme–template preincubation. Similar results were obtained in experiments performed with up to 1 mM compound concentration (data not shown). In contrast, sensitivity of NS5B to inhibition by

3'-deoxy-CTP was not affected by NS5B–RNA complex formation (Fig. 4, left panel).

Discussion

The relative potencies of nucleotide and non-nucleoside inhibitors of HCV RdRp were compared using native HCV replicase and recombinant HCV polymerase, NS5B. As described here, the native HCV replicase showed similar sensitivity to inhibition by 3'-deoxy-CTP as compared to NS5B under similar buffer and substrate incubation conditions. These results suggest that the interaction of NS5B with NTP substrates and NTP analogs is similar in the isolated NS5B protein and the membrane associated replicase complex. Consistent with our finding, triphosphates of 2'-modified nucleoside analogs also inhibited the HCV replicase and the recombinant NS5B with comparable potency (Migliaccio et al., 2003). Many non-nucleoside inhibitors are allosteric inhibitors with binding sites distant from the active site of the RdRp. The potency of this class of inhibitors may depend on binding site accessibility and the conformation of the polymerase. Here, none of the tested representative compounds from three different structural classes measurably inhibited RNA synthesis by HCV replicase even at concentrations more than 1000-fold higher than the concentrations required for half-maximal inhibition of recombinant NS5B (Table 1), although structural and biochemical analyses suggest that these compounds bind to different, non-overlapping binding sites on NS5B protein. Possible explanations for the resistance of the replicase activity to these non-nucleoside inhibitors include (1) different NS5B protein conformation related to productive interaction of NS5B with template RNA, (2) protein–protein interaction between NS5B and other HCV nonstructural proteins or host proteins occluding compound binding sites on NS5B, (3) oligomerization of NS5B during formation of the replicase complex as suggested by *in vitro* studies with

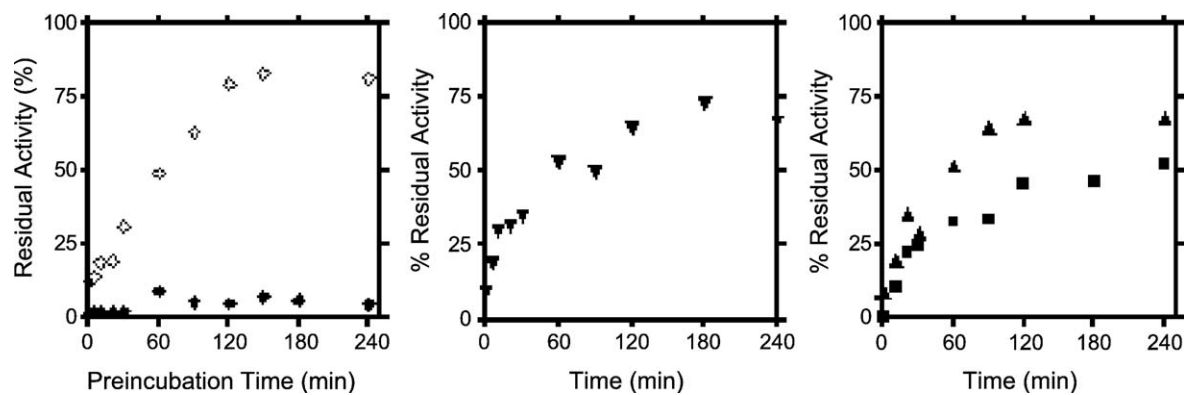


Fig. 4. Effect of RNA template preincubation on the extent of inhibition of NS5B polymerase activity. Recombinant NS5B and cIRES RNA template were preincubated for the indicated time periods before the addition of 1 mg/ml heparin (□), 10 μ M 3'-dCTP (◆), 10 μ M compound 1 (■), 10 μ M compound 2 (▲), and 10 μ M compound 3 (▼). Residual activity in the presence of inhibitor was expressed as a percentage of the RNA synthesis activity in the absence of inhibitor at each preincubation time point.

HCV NS5B or polio virus polymerase (Lyle et al., 2002; Wang et al., 2002). The results obtained in NS5B-RNA preincubation studies support the first hypothesis. Recombinant NS5B protein was protected from inhibition by non-nucleoside as well as heparin after preincubation with template RNA, suggesting that a stable protein–template RNA complex formed during the preincubation period is sufficient to confer the resistance. Similar results were recently reported with related compounds from two different classes of non-nucleoside inhibitors, benzimidazoles and benzothiadiazines (Tomei et al., 2003, 2004). Interestingly, HCV polymerase inhibition by compound 3 from the novel class of thiophene-carboxylic acids, which bind to a thumb region separate from the binding sites of benzimidazole and benzothiadiazine compounds also followed a similar pattern of inhibition as compared to benzimidazoles and benzothiadiazines. Therefore, unexpectedly, compounds binding to three distinct binding sites on NS5B appear to require the opportunity to bind to NS5B prior to formation of a stable protein–template RNA complex to achieve polymerase inhibition. Additional factors may contribute to the high level of replicase resistance to the non-nucleoside inhibitors. The isolated NS5B protein did not achieve full protection from inhibition, even after prolonged preincubation with RNA. The plateau residual activity reached maximal levels of 50–75% after 2–4 h for all three non-nucleosides and heparin, whereas a residual fraction of 25–50% of protein remained apparently sensitive to inhibition. The complete resistance of the native replicase complex as compared to the partial resistance of the protein–RNA complex formed *in vitro* could be related to an increased stability of the polymerase–template complex in the replicase or to the fact that reinitiation of the next round of RNA synthesis is more efficient in replicase complex. Whether increased stability of the protein–RNA complex in the replicase may be conferred by the presence of additional protein–protein or protein–RNA interactions in the multiprotein complex or by the functional oligomerization of NS5B in the replicase complex awaits further studies on the HCV replicase assembly process.

The study described here established that the native HCV replicase complex represents a stable and productive protein–RNA complex with replicative RNA synthesis activity. Allosteric inhibitors of NS5B may therefore function via a common mechanism antagonistically with the formation of a productive protein–template RNA complex, despite binding to distinct, non-overlapping binding sites on NS5B. The previously observed inhibitory effect of these allosteric compounds on HCV replication cell culture therefore most likely results from the inhibition of the formation of new replicase complexes. Discovery of compounds, which could also interfere with the chemical step of NMP incorporation or with NTP substrate binding to NS5B may therefore further increase their overall impact on HCV replication and the inhibitory potency of future non-nucleoside inhibitors.

Materials and methods

Compounds

Non-nucleoside inhibitors were synthesized at Roche Palo Alto according to published procedures. 3′-deoxy-CTP was purchased from TriLink Biotechnologies (#N-3003).

Preparation of cytoplasmic membrane fraction

Cytoplasmic membrane fractions were prepared from Huh7 cells or a Huh7 derived cell line containing an autonomously replicating HCV subgenomic replicon. Cell rupture and cytoplasmic membrane fraction isolation were performed as previously described (Takeda et al., 1986). The isolated membrane fraction was stored in a buffer containing 10 mM HEPES, pH 7.5, 10 mM NaCl, 1 mM DTT, and 15% glycerol at 5×10^5 cell equivalent/ μ l at -80°C until assay.

Protein expression and purification

E. coli strain M15 was used for the expression of HCV NS5B570-BK, which contained an N-terminal histidine tag and a 21 amino acid deletion at the C-terminus. The construct containing the coding sequence of HCV BK strain amino acid residues 2421–2999 (GenBank accession number M58335) downstream of a Taq promoter expression cassette was kindly provided by Hilary Overton, Roche Discovery Welwyn. NS5B570-BK was purified sequentially through Ni-NTA superflow, SP-Sepharose, and Superdex 75 columns. Eluted fractions were checked by SDS-PAGE and homogeneous fractions containing NS5B570-BK were pooled. Protein concentration was determined using Pierce Coomassie Protein reagent.

In vitro replicase assay

The standard replicase assay reactions contained 10 μ l cytoplasmic membrane fraction, 50 mM HEPES, pH 7.5, 10 mM KCl, 10 mM DTT, 5 mM MgCl_2 , 20 $\mu\text{g/ml}$ actinomycin D, 1 mM ATP, GTP, and UTP, 30 μCi [α - ^{33}P]-CTP (3000 Ci/mmol, 10 mCi/ml), 1 U/ μ l SUPERase.In (Ambion), 10 mM creatine phosphate, and 200 $\mu\text{g/ml}$ creatine phosphokinase in a final volume of 25 μ l. The reaction mixtures were incubated at 30°C for 120 min and stopped by the addition of 50 mM EDTA and 0.5% SDS. The RNA products were recovered by phenol–chloroform extraction and ethanol precipitation following removal of proteins by proteinase K digestion. The RNA products were denatured in glyoxal sample loading buffer and resolved in 1% agarose-Sodium phosphate gel. Autoradiographic analysis of the RNA products was performed with phosphorimager and ImageQuant software (Amersham Biosciences). RNA synthesis activity in the presence of inhibitor compounds was expressed as a percentage

of the RNA synthesis activity in the absence of inhibitor compounds.

HCV polymerase assay

The 377-nucleotide-long cIRES RNA template, which was derived from the complementary strand of the IRES region of the HCV genome was generated by T7 RNA polymerase mediated transcription using the MEGAscript T7 Kit (Ambion). Enzymatic reaction mixtures containing 10 µg/ml cIRES RNA template, 200 nM NS5B570-BK enzyme, 2.1 µCi of tritiated UTP (42 Ci/mmol), 1 µM ATP, CTP, and GTP, 1× TMD buffer (40 mM Tris-HCl pH 8.0, 4 mM DTT, and 4 mM MgCl₂), 2.5 mM NaCl, with or without inhibitors in a total volume of 50 µl were incubated for 2 h at 30 °C. Reactions were stopped by the addition of 10% (v/v) trichloroacetic acid. The enzymatic activity of NS5B570 BK was measured as incorporation of radio-labeled nucleotide monophosphates into acid insoluble RNA products. NS5B-RNA preincubation assay was based on the HCV polymerase assay described above with the following modifications. Mixture A (25 µl per reaction) contained 20 µg/ml cIRES RNA template, 400 nM NS5B570-BK enzyme, and 1× TMD buffer. Mixture B (25 µl per reaction) contained 4.2 µCi of tritiated UTP (42 Ci/mmol), 2 µM ATP, CTP, and GTP, 20 µM compound or 2 µg/µl heparin, and 1× TMD buffer. Mixtures A were incubated at room temperature for up to 4 h before the addition of mixture B to start the reaction. The percent relative activity was plotted as a function of preincubation time.

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