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Canonical 3′-deoxyribonucleotides as a chain terminator for HCV NS5B RNA-dependent RNA polymerase

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Abstract

Nucleoside chain terminators represent one of the most promising classes of antiviral drug for DNA viruses and retroviral infection; however, they have not been fully explored against RNA viral polymerases. In this report, we investigate the notion of employing canonical 3′-deoxyribonucleoside triphosphates (3′-dNTPs) as a chain terminator for hepatitis C virus (HCV) NS5B RNA-dependent RNA polymerase (RdRp). Using a HCV RNA transcript-dependent RNA elongating assay, we found that they inhibit NS5B RdRp with K_i ranged from 0.7 to 23 μM. Additional structure–activity relationship studies showed that removal of 2′-hydroxyl group, elimination of ribose's 2′,3′-carbon–carbon bond, or addition of 5-methyl group to a pyrimidine base is detrimental to 3′-dNTP's potency. Direct evidence was obtained that all four canonical 3′-dNTP are incorporated into elongating RNA chains and the incorporation terminates NS5B RdRp-catalyzed RNA synthesis. The K_i values for each of 3′-dNTPs were determined in the single nucleotide incorporation experiments. The nucleoside form of 3′-dNTPs was further evaluated in a cell culture-based HCV subgenomic replicon assay. The discrepancy between the potent in vitro activity and the weak cellular activity of these chain terminators was discussed in the context of nucleoside metabolism. This proof of concept study demonstrates that canonical 3′-dNTPs can function as an effective chain terminator for HCV NS5B RdRp with cytidine as the preferred nucleoside scaffold. Our results further sheds light on the potential hurdles that need to be overcome for successful development of active nucleoside chain terminators in vivo for a viral RNA polymerase, especially the HCV NS5B RdRp.

Keywords: 3′-Deoxyribonucleotide; Hepatitis C virus; NS5B RdRp; Nucleoside chain terminator

1. Introduction

In the past two decades, great progress has been made to discover effective antiviral drugs to control vicious viral infection caused by herpesviruses, human immunodeficiency virus (HIV), and hepatitis B virus (HBV) (Crumpacker, 2001). Notably, 16 unique chemical entities have been developed and approved by US Food and Drug Administration (FDA) for treatment of acquired immunodeficiency syndrome (AIDS) and the related conditions. One prominent class of antiviral drugs is the nucleoside chain terminators that target at viral polymerases. The chemically unique chain terminators that are in clinical use include acyclovir for herpes infection, zidovudine (AZT), didanosine (ddI), zalcitabine (ddC), stavudine (d4T), abacavir (ABC) and tenofovir for AIDS, and lamivudine (3TC) and adefovir (PMEA) for HBV infection. They are all simple pyrimidine or purine nucleosides with modification mainly on the ribose. Strictly speaking, all of these compounds are prodrugs, and intracellular activation is required to convert them to 5′-triphosphate, which is recognized by a viral polymerase. Subsequent incorporation into elongating viral DNA chains terminates viral genome replication due to the fact that these chain terminators lack 3′-hydroxyl group for chain extension. The in vivo phosphorylation of a nucleoside chain terminator is executed by sequential action of as many as three viral or cellular kinases, including orthodox nucleoside kinase, nucleoside monophosphate and diphosphate kinases, and some unconventional cellular kinases. Compared to most of non-nucleoside antiviral drugs that bind to the active site or an allosteric pocket of a viral protein target where substantial drug–protein interactions are established, nucleoside chain terminators behave more like a substrate. Their ability to be incorporated but not extended results in futile viral DNA fragments. Therefore, a chain terminator does not need to form strong and permanent interactions with a viral polymerase, and theoretically drug resistance is less frequent to occur. Since most of the known nucleoside chain

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terminators are designated to against a DNA virus or a retrovirus, they all share a structural feature of 2′-deoxy on the ribose.

A number of RNA viruses such as influenza virus, poliovirus, rhinovirus, and respiratory syncytial virus (RSV) are known human pathogens, and the resultant infections have unmet medical need. Until recently, due to various reasons, little effort has been made to discover specific nucleoside chain terminators for a viral RNA polymerase. HCV is a positive-strand RNA virus. The unraveling HCV as the causative agent for most of non-A and non-B hepatitis cases makes it imminent to develop nucleoside chain terminators for HCV NS5B RdRp (Choo et al., 1989). HCV infection represents major public health hazard with more than 170 million people worldwide and 4 millions in the United States alone being virus carriers (Boyer and Marcellin, 2000). It is the major cause of acute and chronic liver diseases, and a significant portion of which will eventually develop cirrhosis and hepatocellular carcinoma. The current mainstay treatment for HCV is the combination therapy of α-interferon (native or pegylated) with nucleoside drug ribavirin. However, sustained response rate is limited to about 50% with many adverse effects (Davis, 2000). More effective anti-HCV chemotherapy is highly demanded.

We would like to explore the notion of employing ribonucleotides as a chain terminator for HCV NS5B RdRp. The simplest ribonucleotide chain terminators are the four canonical 3′-deoxyribonucleoside triphosphates (3′-dNTP). If a 3′-dNTP can be incorporated into an elongating RNA chain as a substrate, chain termination can be expected due to the missing of 3′-hydroxyl group for further nucleotidyl transfer reaction. Canonical 3′-deoxyribonucleosides have long been synthesized and shown biological or cytotoxic activity against cancer cell cultures (Gitterman et al., 1965; Lin et al., 1991; Walton et al., 1965a,b). They are likely to act through inhibition of cellular RNA synthesis. For example, mode of action studies with 3′-dATP using a partially purified bacterial DNA-dependent RNA polymerase prep indicated that a very limited level of 3′-dATP was incorporated into elongating RNA chains, which caused chain termination for RNA synthesis (Shigeura and Boxer, 1964). In addition, 3′-dNTPs have been shown to be an effective chain terminator for bacteriophage T7, SP6, and ϕ6 RNA polymerase (Axelrod and Kramer, 1985), and employed for RNA sequencing (Makeyev and Bamford, 2001). However, up to date, no solid evidence is available to demonstrate that a canonical 3′-dNTP can be recognized by HCV NS5B RdRp and incorporated into an elongating viral RNA chain as a chain terminator. We set to investigate the feasibility of utilizing 3′-dNTPs as a competitive inhibitor for NS5B RdRp by employing an RNA chain elongation assay. Subsequent single nucleotide incorporation experiments were then applied to seek direct evidence for them to act as a chain terminator.

2. Material and methods

2.1. Material

HCV NS5B was derived from a cDNA clone encoding HCV-1b CON1 strain, and was expressed and purified from *E. coli* as previously reported (Shim et al., 2002). Various [α-^{33}P]rNTPs (3000 Ci/mmol) were purchased from Perkin Elmer. 3′-Deoxyribonucleosides, their 5′-triphosphates and dinucleotide primers were from Sigma, TriLink (San Diego, CA) or ICN Biochemicals. RNA oligonucleotides were custom synthesized by Oligos Etc. (Wilsonville, OR) and PAGE-gel purified. All other reagents were of the highest grade available from ICN, Sigma, Fisher, or Ambion.

2.2. RNA chain elongation assay

A HCV mini-genome of 2.1 kb contains entire 5′-UTR (untranslated region), part of NS5B sequence and entire 3′-UTR was constructed from an internal deletion between two *Kpn*I sites on the HCV replicon plasmid, pFK389/NS3-3′/wt (Lohmann et al., 1999). To generate the mini-genome RNA, the plasmid DNA was linearized with *Ase*I and *Sca*I and transcribed in vitro using MegaScript kit (Ambion, Austin, TX). After phenol–chloroform extraction and isopropanol precipitation, the RNA was resuspended in RNase-free water and stored at −80 °C before use. This HCV CON1 strain-based template was used for the standard NS5B RdRp-catalyzed RNA chain elongation assay.

A standard elongation assay was performed at 23° C in a total volume of 25 μl. The reaction buffer contained 50 mM Tris, pH 7.0, 10 mM $MgCl_2$, 50 mM NaCl, 5 mM DTT (add fresh), and 0.05 mg/ml BSA. A total of 0.4 μg of the RNA template was incubated with NS5B RdRp enzyme (250 nM) before adding mixture of radiolabeled nucleotide (0.2 μCi) and cold nucleotide cocktail to initiate a reaction. The mixture was incubated for 1 h, and the assay was terminated by addition of 75 μl solution of 5% trichloroacetic acid (TCA) and 0.05% pyrophosphate. The quenched solution was incubated at room temperature for 10 min to precipitate out denatured polymeric RNA products, and then subsequently transferred to 96-well white GF/B filter microplate (Packard Instrument) using a Packard Filtermate Universal Harvester. The filter plate was washed five times by water and one time by ethanol before vacuum drying. A total of 40 μl of liquid scintillation cocktail (Packard MicroSint^{TM}) was added to each well. Radioactivity incorporated into the products was counted in 96-well format using a Packard TopCount.

Steady-state kinetic analysis of NS5B RdRp utilizing canonical rNTPs as a substrate was performed in the standard assay conditions as described above. Apparent Michaelis constant K_m for UTP, GTP, or ATP was determined by varying the nucleotide concentration from $K_m/2$ to 10 K_m, and fixing [α-^{33}P]CTP as the radiolabeled nucleotide (13 nM) and the rest two rNTPs at 100 μM. Initial velocity at various nucleotide concentrations was

determined and applied into Michaelis–Menten equation using KaleidaGraph (Synergy Software, Reading, PA) for K_m calculation. The apparent K_m for CTP was measured using [α-^{33}P]GTP as the radiolabeled nucleotide (27 nM) with UTP at 1 μM to minimize wobble base-pairing with guanosine and ATP at 100 μM. All the kinetic experiments described herewith were repeated until consistent results were obtained.

The inhibition studies with 3′-dUTP, 3′-dGTP, or 3′-dATP were performed using [α-^{33}P]CTP as the radiolabeled nucleotide (13 nM), the parental nucleotide substrate at 2 K_m, and the rest two rNTPs at 100 μM. For a specific 3′-dNTP, initial velocity at each inhibitor concentration was determined. Assuming 3′-dNTP only competes with the parental rNTP in a simple competitive mode, then inhibition constant K_i was calculated by plotting $1/v_i$ versus 3′-dNTP concentration using the Dixon plot. For inhibition experiments with 3′-dCTP and analogs, [α-^{33}P]GTP was chosen as the radiolabeled nucleotide (27 nM) with UTP at 1 μM to minimize wobble base-pairing with guanosine, CTP fixed at 120 nM (2 K_m) and ATP at 100 μM.

2.3. Single nucleotide incorporation assay

Well-defined oligo RNA templates were designed to assess the single nucleotide incorporation and inhibition by 3′-dNTP. The oligo templates and dinucleotide primers utilized in these studies are listed in Table 2. A standard single nucleotide incorporation experiment was carried out in 10 μl in a buffer containing 50 mM HEPES (pH 7.3), 10 mM DTT, 5 mM $MgCl_2$. Reaction was started by mixing 20 μM RNA template, 2.5 μM NS5B, 20 μM of ^{33}P-labeled priming dinucleotide or nucleotide (for de novo synthesis), and 1 mM of an elongating nucleotide. The reaction mixture was incubated at 30 °C for 1 h, and then quenched by addition of 10 μl loading buffer (90% formamide, 0.025% bromophenol blue, and 0.025% xylene cyanol). The quenched reaction mixture was heated at 70 °C for 2–5 min prior to loading 2–3 μl onto a denaturing 25% polyacrylamide–7 M urea–TBE gel. Electrophoresis was performed in 1× TBE at 70–90 W. Gels were visualized and analyzed using a PhosphorImager.

Steady-state kinetics of NS5B RdRp utilizing rNTPs as a substrate in the single nucleotide incorporation experiment was examined in the standard conditions as described above. Apparent Michaelis constant K_m for a nucleotide at the fixed concentration of template/primer (20 μM) was determined by varying the nucleotide concentration from $K_m/2$ to 10 K_m value. Initial velocity at each nucleotide concentration was determined and fitted into Michaelis–Menten equation using KaleidaGraph to calculate K_m.

Inhibitory effect of every 3′-dNTP on single nucleotide incorporation was assessed. The experiments were performed in the standard assay conditions as described above with the elongating nucleotide concentration fixed at 1 mM. Initial velocity at various concentrations of a 3′-dNTP was determined, and the inhibition constant K_i for the specific 3′-dNTP was calculated by plotting $1/v_i$ versus inhibitor concentration using the Dixon plot.

2.4. Incorporation of 3′-dNTPs as a chain terminator

To evaluate whether a 3′-dNTP acts as a chain terminator for NS5B RdRp, single nucleotide incorporation experiments were conducted by replacing a particular rNTP with the corresponding 3′-dNTP for substrate incorporation. Further chain elongation was assessed in the presence of other elongation nucleotides. For testing 3′-dGTP as a chain terminator, 20 μM of oligo ribonucleotide 5′-A_5CCAGU-3′ was used as a template and 1 mM of ApC as a primer. A total of 100 μM of [α-^{33}P]UTP was added as the first elongating nucleotide to radiolabel the RNA products. Then, 1 mM of 3′-dGTP was added to the reaction for incorporation into the elongating chain. In the following three experiments where a dinucleotide was utilized as a primer, a dinucleotide primer was first labeled by [γ-^{33}P]ATP using T4 nucleoside kinase following a standard procedure. The final concentration of a dinucleotide in the assay was 20 μM. For evaluation of 3′-dATP as a chain terminator, an oligo template 5′-A_7UGC-3′ (20 μM) was utilized with ^{33}pGpC as a primer. In the reaction, 1 mM of 3′-dATP and UTP were added as the elongating nucleotides. Similarly, for testing 3′-dCTP as a chain terminator, 5′-A_7GAU-3′ (20 μM) was utilized as a template and ^{33}pApU as a primer. In the assay, 1 mM of 3′-dCTP and UTP were added as the elongating nucleotides. For evaluation of 3′-dUTP as a chain terminator, 5′-A_5GGAGC-3′ (20 μM) was utilized as a template and ^{33}pGpC as a primer. In the experiment, 1 mM of 3′-dUTP and CTP were added as the elongating nucleotides. The RNA products were resolved on a 25% polyacrylamide–7 M urea–TBE gel and analyzed by a PhosphorImager.

2.5. Cell-based HCV subgenomic replication assay

We employed a previously reported HCV subgenomic replicon system (Lohmann et al., 1999) to test the inhibitory effect of 3′-deoxy nucleosides on HCV replication in vivo. In the replicon clone (NK/luc-ubi-neo), the structural region of HCV genome is replaced by a fusion gene (*luc-ubi-neo*) containing the luciferase reporter sequence, ubiquitin gene and the neomycin resistance gene. When it is expressed in human hepatoma cells, Huh-7, ubiquitin-mediated proteolytic cleavage generates functional luciferase and neomycin phosphotransferase protein. Huh-7 cells stably transfected with NK/luc-ubi-neo were cultured in DMEM/10% FBS in the presence of 0.5 mg/ml G418. Cells were maintained in a sub-confluent state to ensure high level of HCV RNA synthesis. Luciferase activity was assayed routinely to ensure high level of HCV RNA replication prior to compound testing. For testing a compound's effect on the replicon system, various concentrations of the compound were incubated with cells in a 96-well format at approximately 6000 cells per well. After 48-h incubation at 37 °C, cells were lyzed

and luciferase activity was measured. Reduction in HCV RNA replication activity was calculated based on the reduction of luciferase level and expressed as EC_{50} (effective concentration to reduce 50% of replication). Cytotoxicity of a compound was evaluated by a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent. The cell viability assay was performed using a standard procedure and the results were expressed as CC_{50}. In the experiments, the cell density among various samples was always normalized to determine luciferase activity.

3. Results

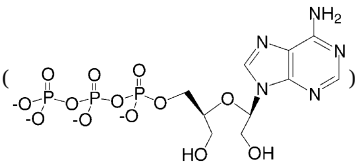
3.1. 3′-dNTP effect on NS5B RdRp from RNA chain elongation assay

To assess the inhibitory effect of 3′-dNTP on NS5B RdRp-catalyzed RNA synthesis, we developed an RNA elongation assay. Using the HCV RNA transcript template described above, NS5B was able to generate a minor product that is the same size as the full-length template and a major product that is almost double size of the template as reported (Alaoui-Lsmaili et al., 2000; Lohmann et al., 1997). While the first product is likely the result of de novo synthesis, the latter product is believed to synthesize through a copy-back mechanism by folding back 3′ end of the template as a primer. An RNA synthesis reaction involves many steps, including initiation, elongation, immature termination, and possibly abortive cycling; therefore, an array of abortive RNA products shorter than full-length of the template are expected. These products were detected in the RNA synthesis assay (data not shown). Due to the process of TCA-precipitation, wash and filtration employed in the assay, only elongated polymeric RNA products were precipitated, retained on the filter and counted. Thus, the radioactivity gauged in this assay should reflect the overall RNA synthesis activity of NS5B RdRp.

Using this elongation assay, the apparent K_m for each canonical rNTP was determined (Table 1). The results are in good agreement with the other reports using a similar assay (Ismaili et al., 2002; Lohmann et al., 2000). Since the apparent K_m values were determined in the RNA synthesis conditions when multiple-rounds of nucleotide incorporation are involved, they may deviate from the intrinsic K_m values from single nucleotide incorporation experiments. Nevertheless, they reflect the apparent utility and affinity of rNTPs as a substrate for NS5B RdRp. The apparent K_m values determined from this chain elongation assay range from 60 nM for CTP to 10 μM for ATP. With the assumption that K_m values reflect the trend of K_d, then the apparent affinity of a rNTP to bind to the NS5B-template binary complex would be in the order of CTP > GTP/UTP > ATP.

3′-Deoxy derivatives of four rNTP substrates were subsequently evaluated as an inhibitor in the RNA elongation assay. By assuming a 3′-dNTP only competes with its parental nucleotide, the apparent K_i was then calculated using the Dixon plot. The K_i values for four 3′-dNTPs are summarized in Table 1. Comparing K_i of a 3′-dNTP to K_m for the corresponding rNTP, removal of 3′-hydroxyl group reduces a nucleotide's apparent affinity to the enzyme complex by 2–25-fold (K_m/K_i). Among the four 3′-dNTPs, 3′-dCTP has the lowest K_i of 720 nM, which likely inherits from the low K_m of CTP (60 nM). Conversely, 3′-dATP has the highest K_i of 23 μM due to the high K_m of ATP.

Table 1
Kinetic parameters of rNTPs and derivatives from RNA chain elongation assay[a]

Nucleotide	K_m (μM)	K_i (μM)	K_i/K_m
UTP	0.24 ± 0.05		
3′-dUTP		5.9 ± 0.5	25
5-Methyl 3′-dUTP		120 ± 20	500
GTP	0.23 ± 0.05		
3′-dGTP		0.93 ± 0.08	4.0
ATP	10 ± 2		
3′-dATP		23 ± 5	2.3
ATP 2′,3′-acyclic dialcohol ()	Not a substrate	Not an inhibitor	
CTP	0.060 ± 0.01		
3′-dCTP		0.72 ± 0.20	12
2′-dCTP		58 ± 8	970
2′,3′-ddCTP		9100	1.5×10^5

[a] Apparent K_m or K_i values for UTP, GTP, ATP and derivatives were determined using [α-^{33}P]CTP as a radiolabeled nucleotide, whereas the values for CTP and its derivatives were determined using [α-^{33}P]GTP as a radiolabeled nucleotide. The kinetic parameters are the average of at least two sets of data.

Table 2
Kinetic parameters of rNTPs and 3′-dNTPs from single nucleotide incorporation assay[a]

Nucleotide	RNA template/primer/radiolabeled NTP	K_m (μM)	K_i (μM)	K_i/K_m
UTP	5′-AAAAACCAGU-3′/ApC/[α-^{33}P]UTP	140 ± 20		
3′-dUTP	5′-AAAAACCAGU-3′/ApC/[α-^{33}P]UTP		110 ± 20	0.80
GTP	5′-AAAAAAACGG-3′/CpC/[α-^{33}P]GTP	67 ± 20		
3′-dGTP	5′-AAAAAAACGG-3′/CpC/[α-^{33}P]GTP		420 ± 120	6.3
ATP	5′-AAAAAAAUGC-3′/^{33}pGpC	130 ± 10		
3′-dCTP	5′-AAAAAAAUGC-3′/GpC/[α-^{33}P]ATP		430 ± 70	3.3
CTP	5′-AAAAAAAAGC-3′/[α-^{33}P]GTP	250 ± 40		
3′-dCTP	5′-AAAAAAAAGC-3′/[α-^{33}P]GTP		410 ± 80	1.6

[a] The kinetic parameters are the average of at least two sets of data.

Subsequent structure–activity relationship (SAR) studies with the 5-methyl derivative of 3′-dUTP indicated that addition of 5-methyl group decreases 3′-dUTP's affinity to the enzyme and increases K_i value by 20-fold (Table 1). This result suggests that as an RNA polymerase, NS5B RdRp strongly prefers uridine to thymidine. Since 3′-dCTP is the more potent than other three canonical 3′-dNTPs in the elongation assay, more SAR was performed on the cytidine scaffold. As illustrated in Table 1, 2′-dCTP has a K_i of 58 μM, indicating that 2′-hydroxyl group is critical for rNTP's recognition. Removal of this group decreases CTP's affinity to the enzyme by almost 1000-fold. 2′,3′-ddCTP was found to have an estimated K_i of 9.1 mM, indicating that the known chain terminator for DNA polymerases, ddCTP, is not recognized by NS5B RdRp. Finally 2′,3′-acyclic dialcohol of ATP was tested in the assay to evaluate the idea of an acyclic nucleotide chain terminator for NS5B RdRp (Table 1). Unfortunately, it was neither a substrate nor an inhibitor for NS5B RdRp (data not shown), indicating that the intact ribose ring is critical for substrate recognition by NS5B RdRp.

3.2. 3′-dNTP effect on NS5B RdRp from single nucleotide incorporation assay

From the chain elongation assay, each of 3′-dNTPs displays inhibitory effect against NS5B RdRp. We would like to determine K_m for each of rNTPs and K_i for their 3′-deoxy counterparts from single nucleotide incorporation experiments. Single nucleotide incorporation assays have been reported for NS5B RdRp using either a dinucleotide as a primer (Zhong et al., 2000a) or through a de novo synthesis mechanism (Shim et al., 2002). For incorporation of canonical GTP, ATP, or UTP as a substrate, a 10-mer oligo ribonucleotide was used as a template and a dinucleotide complementary to the last two bases of 3′ end of a template as a primer (Table 2). In the experiments, addition of a radiolabeled elongating rNTP extended the dinucleotide primer to a 3-mer RNA product, which was subsequently resolved in a denaturing PAGE gel. In the case of CTP, de novo synthesis with a 10-mer template was employed for the purpose (Table 2). Using this single nucleotide incorporation assay, K_m for each of four rNTPs was determined (Table 2). The K_m values range from 67 μM for GTP to 250 μM for CTP, which are at least one magnitude higher than those from the chain elongation assay as described above.

Using the single nucleotide incorporation assay, inhibition experiments with each of 3′-dNTPs were performed. Inclusion of a 3′-dNTP resulted in direct competition with the parental elongating rNTP. Initial velocity at various concentrations of 3′-dNTP was determined, and the apparent K_i value was obtained from the Dixon Plot (Table 2). They vary from 110 μM for 3′-dUTP to 430 μM for 3′-dATP, suggesting that 3′-dNTPs are not potent inhibitors (compared to a substrate's K_m). Purine nucleotides (GTP and ATP) are more sensitive to removal of 3′-hydroxyl group with K_i/K_m ratio from 3.3 to 6.3, whereas two pyrimidine nucleotides are less sensitive with the K_i/K_m ratio of 0.8 to 1.6 (Table 2). Nevertheless, the results suggest that removal of 3′-hydroxyl group does not drastically change the affinity of a rNTP to the enzyme–template complex.

3.3. 3′-dNTPs as a chain terminator

From both RNA chain elongation and single nucleotide incorporation assays, all four canonical 3′-dNTPs were found to capable of competing with rNTP substrates and inhibit NS5B RdRp activity. Further experiments were subsequently performed to seek direct evidence for 3′-dNTPs functioning as a chain terminator. For this purpose, the single nucleotide incorporation experiment described above was modified to incorporate 3′-dNTP in an elongating step. In case of 3′-dGTP, a 10-mer oligo ribonucleotide 5′-A_5CCAGU-3′ was utilized as a template to incorporate 3′-dGTP in the fourth base position. This template retains the last three bases of 3′ end of HCV (+) RNA genome and is capable of initiating de novo synthesis from the +1 position (Shim et al., 2002). Incorporation of 3′-dGTP was originally attempted in de novo RNA synthesis. In the presence of a mixture of ATP, CTP, and [α-^{33}P]UTP, the three nucleotides were incorporated as the first, second, and third base, respectively. Formation of de novo synthesis products pppApC^{33}pU and pppA^{33}pU were observed as expected

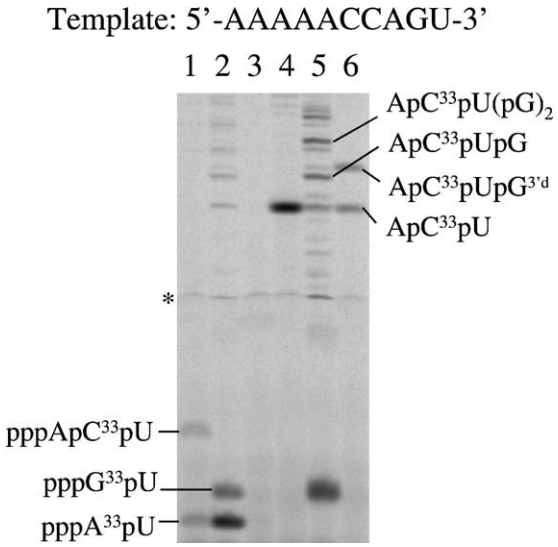


Fig. 1. Incorporation of 3′-dGTP as a chain terminator for NS5B RdRp. In the experiments, oligo 5′-A_5CCAGU-3′ was used as a template. ApC (20 μM) was included as a primer for reactions in lanes 4–6. Each reaction contained 20 μM of template, 2.5 μM of NS5B, 100 μM of [α-^{33}P]UTP, and 1 mM of the following initiating and elongation nucleotide. Lane 1, ATP and CTP; lane 2, ATP, CTP, and GTP; lane 3, ATP, CTP, and 3′-dGTP; lane 4, ApC only; lane 5, ApC and GTP; lane 6, ApC and 3′-dGTP. The reaction products were resolved on a 25% polyacrylamide–7 M urea–TBE gel and were analyzed by a PhosphorImager. Asterisk denotes gel artifact.

(Fig. 1, lane 1). The latter product was from the formation of wobble base-pair between G and U. Inclusion of GTP in the elongation process extended the initial products to a ladder of elongating products plus pppG^{33}pU, a product of wobble base-pair formation between G and U (lane 2). Interestingly, addition of 1 mM 3′-dGTP in the reaction totally inhibited the de novo synthesis with no RNA elongating product observed (lane 3), indicating that 1 mM of 3′-dGTP indeed suppresses the RNA synthesis as predicted from the previous inhibition studies. In this case, 3′-dGTP is likely to form a wobble base-pair with U in the 3′-end of the template, and thus stops RNA synthesis in the initiation phase. Subsequently, 3′-dGTP was tested in the dinucleotide-dependent RNA synthesis assay to bypass the initiation step. In this experiment, the same RNA template was employed with ApC as a primer. In the presence of [α-^{33}P]UTP, a trinucleotide product ApC^{33}pU was observed as expected (Fig. 1, lane 4). Inclusion of GTP resulted in formation of the elongating products ApC^{33}pU, ApC^{33}pUpG, ApC^{33}pUpGpG and a ladder of longer products (lane 5). Conversely, replacement of GTP with 3′-dGTP gave only two RNA product bands with the previously observed ladder of elongating products completely abolished (lane 6). Based on the mobility, the two radiolabeled products were assigned as ApC^{33}pU and ApC^{33}pUpG$^{3'd}$. Thus, 3′-dGTP was indeed incorporated into the elongating chains as a chain terminator.

The rest three canonical 3′-dNTPs were evaluated in the similar experiments. For 3′-dATP, a 10-mer template 5′-A_7UGC-3′ and ^{33}pGpC dinucleotide primer were designed to detect incorporation of 3′-dATP in the third base position. In the presence of elongation nucleotides ATP and UTP, a ladder of elongating products was observed as expected (Fig. 2A, lane 1). However, by replacing ATP with 3′-dATP, the ladder of elongating products disappeared, and a new band with mobility slower than dinucleotide ^{33}pGpC was observed (lane 2). The new product was assigned as^{33}pGpCpA$^{3'd}$. Clearly, 3′-dATP was incorporated into the elongating chains as an effective chain terminator.

For pyrimidine ribonucleotide 3′-dCTP, a 10-mer template 5′-A_7GAU-3′ and dinucleotide primer ^{33}pApU were utilized for the incorporation experiment. In the presence of CTP and UTP, a ladder of elongating products starting from ^{33}pApUpC was detected (Fig. 2B, lane 3). Replacement of CTP by 3′-dCTP resulted in formation of single new product with mobility slower than dinucleotide ^{33}pApU (lane 4), which was assigned as ^{33}pGpCpA$^{3'd}$. Indeed 3′-dCTP was incorporated into the elongating chains, and the incorporation terminated the further chain extension.

For evaluating incorporation of 3′-dUTP as a chain terminator, a 10-mer template 5′-A_5GGAGC-3′ and dinucleotide primer ^{33}pGpC were used. In the presence of UTP and CTP, a ladder of elongating products starting from ^{33}pGpCpU was generated (Fig. 2C, lane 3). Replacement of UTP by 3′-dUTP resulted in formation of a single product with mobility slower than dinucleotide ^{33}pGpC (lane 4). This new product was assigned as ^{33}pGpCpU$^{3'd}$. Clearly NS5B recognized 3′-dCTP as a substrate and the 3′-dCTP incorporation stopped further chain elongation.

3.4. 3′-Deoxyribonucleoside's effect on HCV subgenomic replication activity

Following in vitro enzymatic activity assay, the nucleoside form of four canonical 3′-dNTPs was evaluated in a HCV subgenomic replicon system that carries a luciferase reporter gene (Lohmann et al., 1999). Reduction in HCV RNA replication activity was observed for all the tested compounds (data not shown), and EC_{50} value was determined (Table 3). Compared to the in vitro activity, these

Table 3
Inhibitory and toxic effects of various ribonucleosides from Huh7-based HCV subgenomic replicon assay

Nucleoside	EC_{50} (μM)[a]	CC_{50} (μM)
3′-Deoxyuridine	200	>300
5-Methyl 3′-deoxyuridine	200	>300
3′-Deoxyguanosine	500	>300
3′-Deoxyadenosine	150	>300
3′-Deoxycytidine	45	>300
2′-Deoxycytidine	200	>300
2′,3′-Dideoxycytidine	200	>300
Cytidine	200	>300

[a] The EC_{50} results carry less than 20% error rate.

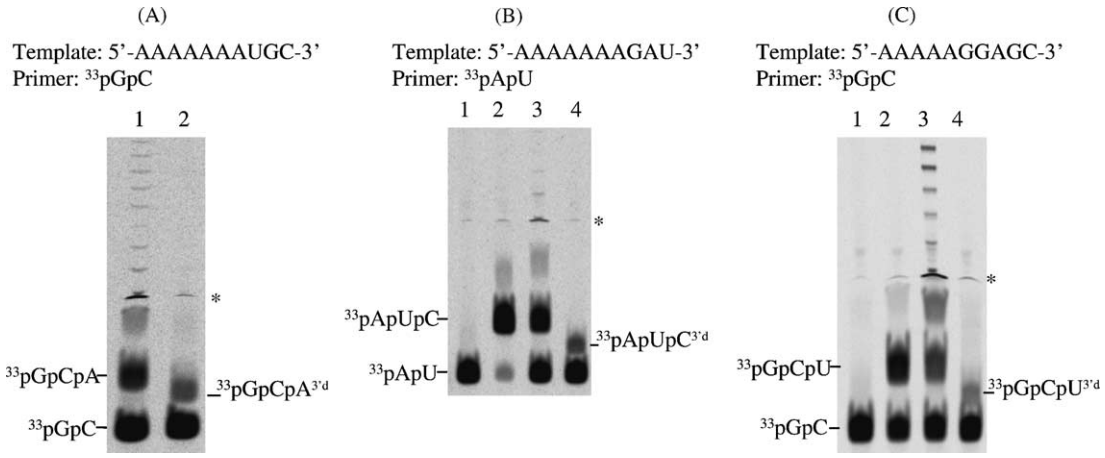


Fig. 2. Incorporation of 3′-dNTPs as a chain terminator for NS5B RdRp. In the experiments, a 10-mer oligo (20 μM) was used as a template with a dinucleotide (20 μM) as a primer. The template and primer sequences were depicted above each gel. All the elongation nucleotide concentrations were fixed at 1 mM. The reaction products were resolved on a 25% polyacrylamide–7 M urea–TBE gel and were analyzed by a PhosphorImager. Asterisk denotes gel artifact. (A) For incorporation of 3′-dATP, the following elongation nucleotides were added to respective reactions: lane 1, ATP and UTP; lane 2, 3′-dATP and UTP. (B) Incorporation of 3′-dCTP as a chain terminator. The following elongation nucleotides were included in the following reactions: lane 1, ^{33}pApU only; lane 2, CTP; lane 3, CTP and UTP; lane 4, UTP and 3′-dCTP. (C) Incorporation of 3′-dUTP as a chain terminator. The following elongation nucleotides were added to each reaction: lane 1, ^{33}pGpC only; lane 2, UTP; lane 3, CTP and UTP; lane 4, CTP and 3′-dUTP.

compounds are much weaker inhibitors in the replicon system. Among the four nucleosides, 3′-dC is the most potent one with EC_{50} of 45 μM, whereas 3′-dA, 3′-dG, and 3′-dU have EC_{50}s of 150, 200, and 200 μM, respectively. Subsequent titration experiments with cytidine analogs indicated that either of 2′-dC, 2′,3′-ddC, or cytidine has an EC_{50} value around 200 μM (Table 3). These results suggest that any compounds with EC_{50} above 200 μM do not have any specific inhibitory effect on the replicon system. Finally, cytotoxicity of these nucleosides was evaluated by MTT cell viability assay. None of the tested nucleosides has cytotoxic effect on the Huh-7 cells up to 300 μM (Table 3).

4. Discussion

In this proof of principle study, we set to investigate canonical 3′-dNTPs as a chain terminator for a viral RNA polymerase, HCV NS5B RdRp. For a nucleotide to serve as an effective chain terminator, it has to be accepted as a substrate and get incorporated into an elongating chain. Although one may foresee NS5B RdRp should utilize 3′-dNTPs as a substrate, the effect on substrate recognition by removing 3′-hydroxyl from the ribose is unpredictable. 3′-Hydroxyl group is believed to interact directly with the catalytic Mg^{2+} ion in the polymerase active site and is responsible for stabilizing the pentavalent intermediate on the α-phosphate during catalysis (Gohara et al., 2000; Tabor and Richardson, 1995). Using an RNA elongation assay, we demonstrate that all four canonical 3′-dNTPs inhibit NS5B RdRp with K_i values ranged from 0.7 to 23 μM (Table 1). The preferred nucleoside scaffold is CTP, which has the lowest K_m, and its 3′-deoxy derivative has the most potent K_i. Considering the extremely high concentration of cellular rNTPs that a chain terminator has to overcome in vivo, it would be critical to choose the best nucleoside scaffold for designing a chain terminator. Since the cellular concentration of rNTPs is in the order of ATP > GTP ≥ UTP > CTP (Traut, 1994), it is predicted that cytidine-based chain terminators would have a better chance to inhibit HCV NS5B RdRp in vivo. This prediction coincides with the results from the HCV subgenomic replication assay that 3′-dC is the most potent inhibitor among the four tested canonical 3′-deoxyribonucleosides.

A nucleic acid polymerase is able to discriminate substrate structure so as to specify type of synthesis of DNA, RNA, or nucleic acid with non-canonical nucleotides. The SAR studies using the chain elongation assay indicate that HCV NS5B RdRp possesses all the distinct substrate requirements of an RNA polymerase. The considerable K_i difference between 3′-dUTP and 5-methyl 3′-dUTP suggests that NS5B RdRp prefer uridine to thymidine. The drastic increase of K_i by removal of 2′-hydroxyl group from 3′-dCTP indicates that 2′-hydroxyl group plays a critical role in substrate recognition by NS5B RdRp. The recent studies of NS5B–rNTP complex crystal structure explained the importance of 2′-hydroxyl group in substrate specificity (Bressanelli et al., 2002). 2′-Hydroxyl group of a UTP in the NS5B co-crystal structure was found to form a hydrogen bond with residue Asp225 of the conserved motif A. Although the reported NS5B complex structure lacks the template in the active site, it is believed that this hydrogen bond will be preserved in the NS5B productive complex. By comparison, no specific interaction was observed for 3′-hydroxyl group in the crystal structure. This is

consistent with our observation that removal of 3′-hydroxyl group only reduces a rNTP's affinity by a modest level based on K_i/K_m ratio, whereas elimination of 2′-hydroxyl group from 3′-dCTP almost totally wipes out its affinity to the enzyme complex. Finally we demonstrate that an acyclic analog of ATP is neither a substrate nor an inhibitor, suggesting an intact ribose ring is imperative for substrate recognition. These results implicate that the 2′-hydroxy and the intact ribose ring may have to be preserved for a potent chain terminator.

The inhibitory effect of 3′-dNTPs on NS5B RdRp is also confirmed in the single nucleotide incorporation experiment. However, the K_i values from the assay are a lot higher than those determined from the RNA elongation assay. Due to the complexity of a polymerase reaction, the K_i values from the two assays may not be comparable. First, the two assays have very different readouts. The elongation assay involves multiple rounds of rNTP incorporation where a 3′-dNTP may compete with as many as four rNTPs for incorporation in every single round. The final readout reflects the global effect of an inhibitor on RNA synthesis, and the results are more physiologically relevant. On the other hand, in the single nucleotide incorporation assay, only one round of nucleotide incorporation is executed and a 3′-dNTP only contests with its parental rNTP. This K_i should better represent the intrinsic affinity of a nucleotide to the enzyme–template complex. Second, the two assays utilize very different templates. The elongation assay uses a 2.1-kb HCV genome as a template, whereas the single nucleotide incorporation assay utilizes an artificial 10-mer ribonucleotide. The higher K_i values from single nucleotide incorporation assay may indicate a weak productive enzyme–template complex. Although solid evidence is lacking, HCV is believed to undergo de novo RNA synthesis like most of known RNA viruses. Recombinant NS5B RdRp was shown to be able to initiate de novo RNA synthesis and yield a full-length HCV RNA genome (Oh et al., 1999). A minimal 98 nucleotide (nt) from 3′-end of the (+)-strand or 239 nt from 3′-end of the (−)-strand HCV RNA genome is required to serve as a template for efficient de novo RNA synthesis, indicating the presence of *cis*-acting sequences for HCV RNA synthesis. Short artificial ribonucleotides as short as 4-mer are utilized as a template for HCV de novo synthesis; however, the polymerase activity is lower, and immature chain termination and possibly abortive cycling were observed during elongation (Shim et al., 2002; Zhong et al., 2000b). These studies lead to a conclusion that efficient de novo RNA synthesis requires specific template sequences and structural elements recognized by NS5B RdRp. To detect single nucleotide incorporation, a short oligo template has to be used. But a short template lacks the necessary structural elements for higher RdRp activity. Consequently the observed NS5B RdRp activity is lower. Therefore, the apparent K_m for a nucleotide substrate would be higher in the single nucleotide incorporation experiments. Our results from the single nucleotide incorporation experiments are in line with the prediction. Due to the same reason, the K_i values for 3′-dNTPs in the single incorporation assay are higher than those from the elongation assay. In addition, the different template sequences and primers employed in the single nucleotide incorporation experiments may contribute to the variation of kinetic parameters among four rNTPs (for K_m) and 3′-dNTPs (for K_i), which makes it hard to compare the relative utility and affinity of four nucleotides. In conclusion, the functions of two NS5B RdRp assays are complementary. The chain elongation assay is useful to assess the global effect of a substrate or an inhibitor on RNA synthesis. Conversely, the single nucleotide incorporation experiments are indispensable for determining intrinsic kinetic properties of a substrate or an inhibitor and for obtaining direct evidence for a nucleotide to act as a chain terminator.

We tested four canonical 3′-deoxyribonucleosides in a HCV subgenomic replication assay. Intriguingly, their EC_{50} values are at least 10-fold higher than the K_i values of the corresponding 3′-dNTPs from the elongation assay. A nucleoside is a pro-drug and its activity depends on cellular kinases to convert it to 5′-triphosphate. Therefore, lack of phosphorylation could contribute to the weak cellular activity. Moreover, these simple nucleoside chain terminators have to overcome the extremely high concentration of cellular rNTPs for incorporation into viral RNA chains. The versatile nucleoside metabolism such as deamination, dephosphorylation, and base cleavage could reduce their inhibitory potency as well. Experiments are in progress to study the 3′-deoxyribonucleoside metabolism in the cells to fully address the discrepancy between the in vitro and cellular activities of these chain terminators.

In summary, we tested the notion of utilizing canonical 3′-dNTPs as a chain terminator for HCV NS5B RdRp. All four 3′-dNTPs were found to inhibit NS5B RdRp activity. But comparing its K_i to the K_m of a rNTP, a canonical 3′-dNTP's affinity to NS5B RdRp is similar to a substrate's. Single nucleotide incorporation assays demonstrate that all four 3′-dNTPs can be incorporated like a substrate and function as a chain terminator. Cell culture testing of the corresponding nucleosides reveals that they are weak inhibitors for HCV genome replication. Although canonical 3′-dNTPs can function as an effective chain terminator for NS5B RdRp in vitro, their potency has to be further improved in order to be effective in vivo. Our kinetic analysis and structure–activity relationship studies lay the foundation to select preferred nucleoside scaffold for further modification to make potent chain terminators for viral RNA polymerases.

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