Nucleoside Analog Inhibitors of Hepatitis C Virus Replication

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Abstract: Of the 30 compounds currently marketed in the United States for treatment of viral infections, 15 are nucleoside analogs, demonstrating the utility of this class of compound as a source of antiviral drugs. The success of nucleoside analogs in treating other viral infections provides a compelling rationale for the significant effort that is currently being devoted to the discovery and development of nucleoside analogs to treat infection by hepatitis C virus (HCV) that may lead to improvements in response rates compared to currently available therapies. Several different approaches have been adopted to identify promising analogs, including the use of surrogate viruses in cell culture assays, screening in the cell-based bicistronic HCV replicon assay, and screening nucleoside triphosphates for the ability to inhibit the activity of the HCV RNA-dependent RNA polymerase *in vitro*. Several classes of ribonucleoside analogs with modifications of the ribose inhibit HCV replication. Nucleoside analogs incorporating a 2'-C-methyl modification are potent inhibitors in the replicon assay in the absence of cytotoxicity, and appear to exert their inhibition by acting as functional chain terminators of RNA synthesis. NM283, a prodrug of 2'-C-methylcytidine, has entered clinical trials and demonstrated viral load reductions in subjects infected with genotype 1 HCV, a genotype known to be difficult to treat effectively with currently approved therapies. Overall, results to date offer encouragement that improved therapies to treat HCV infection including newly developed nucleoside analogs may become available within the next few years.

Keywords: Nucleoside analog, replicon, structure-activity relationships, RNA polymerase, chain terminator.

HCV BACKGROUND

HCV[1] was recognized as the infectious agent responsible for community-acquired non-A non-B hepatitis in 1989 [1]. This discovery made possible the development of diagnostic tests for HCV that have reduced the risk of infection through blood transfusion. Yet, estimates of the total number of infected individuals are currently 170-200 million worldwide [2]. HCV infection is the leading cause of liver transplantation in the United States. Currently preferred therapies to treat HCV infection consist of six to twelve month courses of combinations of pegylated interferon α and ribavirin, which result in sustained viral response (SVR, no detectable virus six months after cessation of therapy) in 40-60% of treated patients [3, 4]. The SVR rates with patients infected with genotype 1 virus, the predominant genotype in most western countries, are lower than with genotype 2 infections [3, 4]. The low SVR coupled with the frequency of side effects associated with interferon-ribavirin combination therapies necessitates the development of improved therapies to treat HCV infection.

HCV is a positive-stranded RNA virus of the family Flaviridae, which also includes human pathogens yellow fever, West Nile, and dengue viruses. Replication of the HCV genome is catalyzed by a complex of virally-encoded and potentially cellular [5] proteins. At the heart of the replication complex is the HCV RNA-dependent RNA polymerase (RdRp) which is responsible for catalyzing ribonucleotide incorporation leading to the formation of both the negative strand copy of the viral genome and subsequent positive strand copies that serve as the genomes of progeny virus. Since it is absolutely required for viral infectivity [6], HCV RdRp is a validated and attractive target for the development of new treatments for HCV infection based on administration of compounds that directly inhibit viral enzyme function.

Extensive structural information is now available for HCV RdRp including co-crystals with some non-nucleoside inhibitors [7-11]. As with other polymerases such as the Klenow fragment of E. coli DNA polymerase I and HIV reverse transcriptase, the overall structure of HCV RdRp has been compared to a right hand with fingers, palm and thumb subdomains. Unique to the HCV RdRp, though, are extended finger domains that come into contact with the thumb leading to a completely encircled active site, which includes catalytic aspartic acids 318 and 319. The dynamics of the contacts between the fingertips and the thumb have been implicated in the mechanism of action of some non-nucleoside inhibitors [12]. The C-terminal 21 amino acids are highly hydrophobic and likely form a membrane anchor [13]. Deletion of the C-terminal tail gives rise to a more soluble enzyme which is utilized in many *in vitro* studies [14].

NUCLEOSIDES AS THE BASIS FOR ANTIVIRAL THERAPIES

Nucleoside analogs are successfully employed to treat infections with HIV, hepatitis B virus, and herpes viruses, all of which encode a polymerase whose primary activity is DNA synthesis. All of the nucleoside drugs used to treat these infections can be considered deoxynucleoside analogs.

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HCV RdRp as an RNA polymerase might be expected to exhibit different structural requirements for nucleoside analogs that would function as inhibitors, particularly at the 2'-position of the ribose where inclusion of a hydroxyl would likely be advantageous for recognition by RdRp. The presence of a 2'-hydroxyl group on the nucleoside offers a chance for greater selectivity of inhibition of the HCV RdRp over cellular DNA polymerases. Inhibition of the mitochondrial DNA polymerase gamma by deoxynucleoside analog triphosphates is thought to be the cause of mitochondrial toxicity associated with the administration of some nucleoside analogs, for example, ddC [15, 16]. Mitochondrial toxicity is likely the basis for the hyperlactatemia, lactic acidosis, steatosis, peripheral neuropathy, myopathy, cytopenias, pancreatitis, and lipoatrophy that are associated with long term use of nucleoside inhibitors of HIV reverse transcriptase [17]. However the use of a ribonucleoside analog could also open the door to toxicities associated with interfering with one or more of the many other roles that ribonucleosides play *in vivo* – in inter- or intracellular signaling, energy storage and use, protein modification, or cellular RNA synthesis.

The overall requirements for an effective chain-terminating nucleoside analog inhibitor for oral treatment of HCV infection are numerous. First, the analog must be absorbed efficiently from the gastrointestinal tract. Nucleoside transporters expressed in the intestinal epithelium that are responsible for the uptake of most nucleoside analogs have been described [18]. In contrast, passive diffusion appears to be important for acyclovir. Prodrug approaches to improving the oral bioavailability of nucleosides have made use of amino acid ester modifications, for example valacyclovir, that appear to allow transport by the oligopeptide transporter pepT1 and others [18, 19].

Additionally, the analog must have acceptable pharmacokinetic (PK) parameters, have a long half-life sufficient preferably for once or twice a day dosing, and achieve significant concentrations in the target organ, liver. In this sense standard plasma PK analysis, though useful in determining the overall exposure to a compound, can actually be misleading. A compound that can achieve excellent uptake into the liver may appear to have poor exposure in plasma in a short-term analysis, since little remains in the circulation. In other words, a high first-pass uptake into liver is desirable in this case. Balancing the need for high concentrations of the nucleoside in liver is the general requirement that since drug exposure is typically monitored in plasma in the clinical setting, some plasma exposure is also required. Optimally, the ratio of compound concentration in liver to that in plasma will be constant across time and species, so that the level of compound in plasma will be predictive of liver concentrations when the compound is dosed in human subjects.

Once it has been absorbed into blood the analog must enter hepatocytes, either through nucleoside or other transporters or by passive diffusion, as shown in Fig (**1**). The

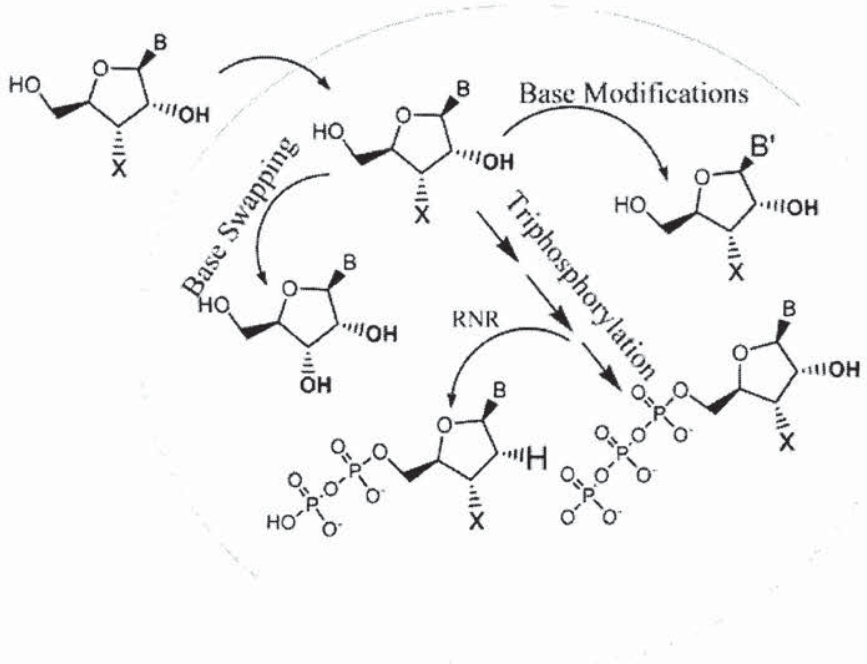

Fig. (1). Intracellular metabolism of nucleoside analogs. A ribonucleoside analog with a modified ribose enters a cell *via* nucleoside transporters, passive diffusion, or peptide transporters. In the intracellular environment, the nucleoside can either undergo 5'-phosphorylation to the monophosphate by the action of a nucleoside kinase, conversion to a base modified form (B') *via* nucleoside metabolizing enzymes such as adenosine deaminase, or "base-swapping" *via* purine nucleoside phosphorylase which reversibly catalyzes the cleavage of the glycosidic bond. The monophosphate is converted to the diphosphate *via* nucleotidyl kinase. The diphosphate may be converted to a deoxyribose diphosphate by ribonucleotide reductase, which may then be incorporated into cellular DNA. Alternately the disphosphate may be converted to the triphosphate by nucleoside diphosphate kinase.

roles of equilibrative and concentrative transporters in the uptake and efflux of nucleoside analogs from cells have been recently reviewed [20]. The analog must then serve as a substrate for three intracellular kinases to be converted to the active 5'-triphosphate, in general without undergoing unwanted metabolism through the activity of other nucleos(t)ide metabolizing enzymes. Modification of an adenine base by adenosine deaminase, for example, would form the inosine analog, which may not be inhibitory to polymerase activity. Cleavage of the glycosidic bond through the activity of purine nucleoside phosphorylase (PNP) can result in scrambling of a modified ribose with other nucleobases. In particular, conversion of the diphosphate to a deoxynucleoside diphosphate *via* ribonucleotide reductase is undesirable for nucleobase-modified analogs, since that would allow for possible incorporation into cellular nuclear DNA which may lead to mutagenesis and carcinogenesis. For nucleoside analogs intended to treat hepatitis, an important consideration is the expression pattern of transporters and the nucleoside metabolizing enzymes specific to hepatic tissue which is characterized by high catabolic rates [21].

Once the 5'-triphosphate of the analog is generated, it must serve specifically as a substrate for the HCV RdRp and not as a substrate for cellular RNA polymerases and be incorporated into HCV genomic RNA. Incorporation of the nucleotide analog must take place against a background of competing natural nucleoside triphosphates that are present at concentrations that range from hundreds of micromolar for CTP, UTP and GTP to millimolar concentrations for intracellular ATP [22]. In contrast the concentrations of deoxynucleoside triphosphates are generally 10-fold lower. Thus, effective inhibition through incorporation of a chain terminator may be more difficult to achieve in the intracellular environment with an RdRp than with a DNA polymerase. Once incorporated, the analog should no longer support further RNA synthesis by preventing the addition of more nucleotides. In the absence of a mechanism for excision of the incorporated chain terminator, the truncated viral genome would be unable to support further rounds of RNA synthesis. Although it has not been directly demonstrated, since HCV RdRp catalyzes the incorporation of ribonucleotides with concomitant release of pyrophosphate, it must also catalyze the reverse reaction, pyrophosphorolysis. It remains to be seen whether such an excision mechanism operates in cells and under what conditions. These multiple requirements for effective nucleoside analog inhibitors present a real challenge to drug development.

Despite these challenges, several different nucleoside analogs that inhibit HCV replication as measured in the replicon assay have been discovered. The majority of the published information is available for the class of nucleoside analogs containing a 2'-C-methyl substituent. Recent reviews of progress in the discovery and development of other classes of inhibitors of HCV replication are available [23-26]. The proposed mechanism of ribavirin notwithstanding, this review will focus on novel nucleoside analogs that inhibit HCV RdRp.

DISCOVERY OF 2'-C-METHYL MODIFIED ADENOSINE ANALOGS

In the past, the inability to propagate HCV robustly in cell culture necessitated the use of surrogate assays to screen for inhibitors of viral replication. Bovine viral diarrhea virus (BVDV) is a pestivirus within the family *Flaviviridae*, with homology to HCV that has been used as a surrogate, since it is easily propagated in cultures of MDBK cells, and does not infect humans. The use of BVDV antiviral assays for investigating inhibitors has primarily been replaced with assays based on the HCV replicon [27]. Recently, cell culture systems capable of propagating HCV have been developed [28-30] that will likely replace the replicon system for routine compound screening.

The overall level of homology between the RNA polymerases of HCV and BVDV, even when ignoring an N-terminal domain of BVDV RdRp that is not found in the HCV enzyme, is quite low. However, within the active sites of the polymerases, the homology is much stronger, suggesting that BVDV might be a useful surrogate for HCV for discovery of active-site directed inhibitors, such as nucleoside analogs. Confounding this approach, though, would be the use of MDBK cells to propagate BVDV which might have different capabilities for converting nucleoside analogs to the active 5'-triphosphates than human hepatic or hepatoma cells [31]. The low overall homology between HCV and BVDV RdRp suggests that searches for non-active site directed compounds analogous to the non-nucleoside reverse transcriptase inhibitors of HIV might not be successful. This lack of correspondence has been borne out experimentally by the identification of non-nucleoside inhibitors (NNIs) that are BVDV- or HCV-specific [10, 32-37].

2'-C-Methyl nucleosides were identified as potent inhibitors of BVDV replication in cell culture by screening libraries of nucleoside analogs (R. LaFemina, personal communication; [38]). Subsequently it was shown that the 5'-triphosphates of 2'-C-Me-nucleosides can inhibit the catalytic activity of both BVDV and HCV RdRp *in vitro*. In parallel to screening nucleosides in BVDV antiviral cell culture assays, chemically synthesized nucleoside triphosphates were screened for inhibition of purified HCV RdRp. The *in vitro* screens for HCV RdRp inhibitors aided in the understanding of some aspects of the structure-activity relationships for inhibition of the target enzyme.

STRUCTURE-ACTIVITY RELATIONSHIPS OF 2'-MODIFIED NUCLEOSIDE ANALOGS

Structural modifications to the ribose and nucleobase have delineated some aspects of the requirements for efficient inhibition in both the RdRp and cell-based replicon assay. In general the results indicate that a very narrow range of substituents gives rise to potent inhibition, particularly in the replicon assay, owing in large part to the multiple structural requirements for efficient uptake of the nucleoside into the cell, conversion to the 5'-triphosphate, and the absence of unwanted metabolic conversion to inactive analogs, that are necessary in order to inhibit viral RNA replication in the cellular environment.

Modifications to the ribose have investigated the size, hydrophobicity, electronics, and regiospecificity of substituents, with the focus on modifications at the 2' and 3' positions, as shown in Fig. (**2**). Conversion of the 2'-C-methyl substituent of the adenosine analog (**1**) to 2'-C-ethyl (**2**) completely abolishes inhibition by the nucleoside in the replicon assay, and by the corresponding 5'-triphosphate in the enzyme assay [39]. Modeling of the ethyl substituted analog into the enzyme active site in the position that corresponds to the substrate nucleoside triphosphate suggests that where the 2'-C-methyl modification can be accommodated into the enzyme active site, the larger ethyl modification would clash sterically with the side chain of Ser282, analogously to the steric clash between the 2'-C-methyl substituent with the methyl group of the resistance mutation Ser282Thr, as discussed below.

The importance of the stereoelectronic character of the 2'-C-methyl to inhibition was investigated by conversion of the methyl group to either CH_2F (**3**) or to CF_3 (**4**) [39]. In the case of CH_2F, a 16-fold reduction in inhibitory potency in the replicon assay was observed, whereas the CF_3 substitution led to abolishment of cell-based activity. The stereospecificity for inhibition was examined by inverting the configuration at the 2'-position. The inverted analog (**5**) was not inhibitory either as the triphosphate in the enzyme assay or as the nucleoside in the replicon assay, suggesting the importance of maintaining the ribo configuration at the 2'-carbon [39]. The critical importance of retaining the 3'-hydroxyl to inhibitory potency is demonstrated by the lack of activity of the 3'-deoxy-2'-C-methyladenosine analog (**6**) in both the enzyme and replicon assays. Though HCV RdRp does not make use of the 3'-hydroxyl of 2'-C-methyl nucleotides as a nucleophile during extension as discussed below, the 3'-hydroxyl likely serves as an important binding determinant for the initial incorporation event that leads to formation of the analog-terminated primer. Moving the methyl substituent from the 2'-carbon to the 3'-carbon (**7**) also leads to complete loss of inhibitory potency [39].

Exploration of the structure-activity relationships of the nucleobase reveals modifications that enhance inhibitory potency. 2'-C-Methylcytidine (**8**) and 2'-C-methylguanosine (**9**) were both active in the replicon assay though with reduced potency relative to 2'-C-methyladenosine, suggesting that the identity of the base is not critical to inhibition [40]. The reduced inhibitory potency relative to the adenosine analog is a consequence of deleterious

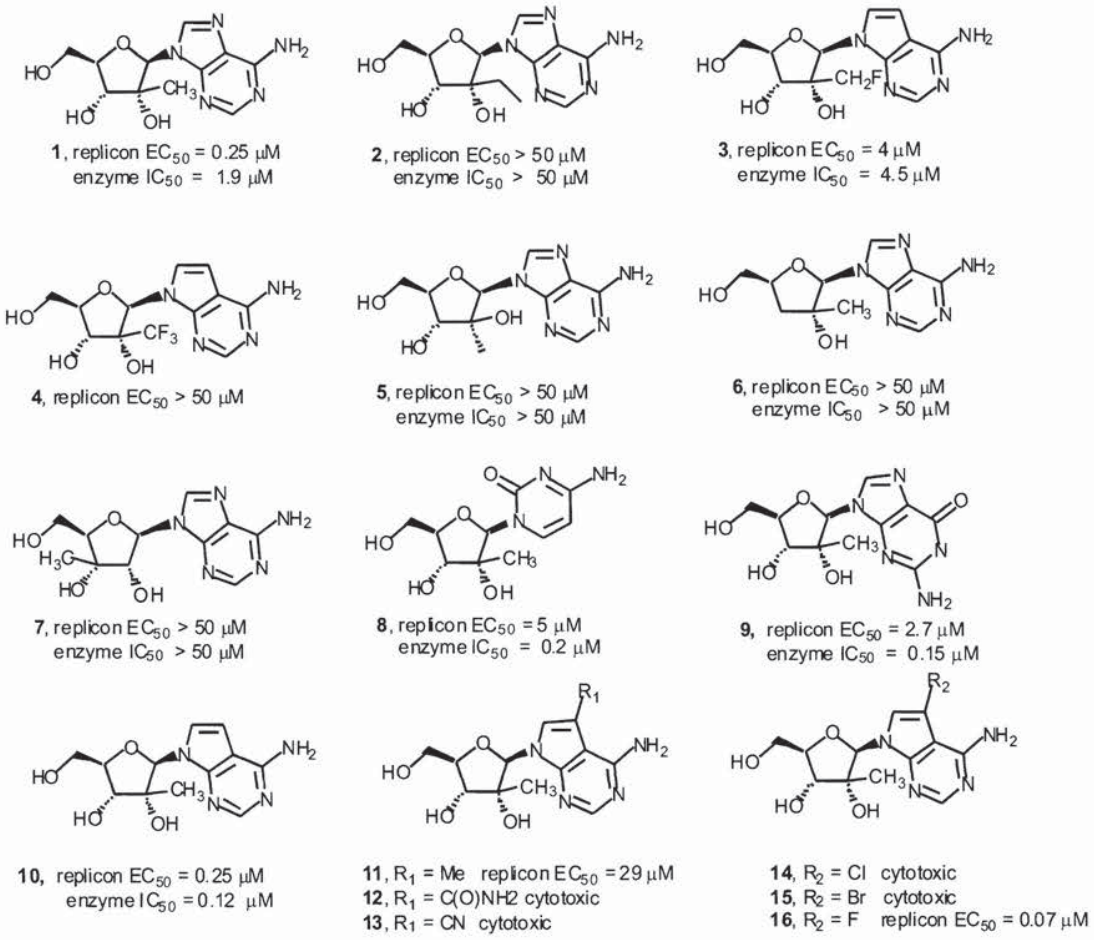

Fig. (2). Structure activity relationships for inhibition of HCV RdRp by nucleoside analogs.

metabolism to the inactive uridine analog in the case of 2'-C-methylcytidine, and a reduction in cellular uptake and/or efficiency of kinasing in the case of the guanosine analog. One of the most useful modifications of the base that has been identified is the 7-deaza-purine substitution. The 7-deaza modification was first identified as a potency-enhancing substitution within the context of the enzyme assay. By comparison of IC_{50} values for inhibition in the enzyme assay for pairs of nucleoside triphosphates with either nitrogen or carbon at the 7-position of either adenine or guanine-containing analogs and with several different ribose modifications, a consistent 10-20-fold increase in inhibitory potency was observed for the 7-deaza version, demonstrating that the 7-deaza modification is beneficial to inhibitory potency independent of the nature of the ribose modification [41]. In fact, the substrate efficiency of ATP compared to that of 7-deaza-ATP (tubercidin triphosphate) also demonstrated a 10-fold improvement for the 7-deaza-modified triphosphate, suggesting that the improved recognition by the enzyme for the 7-deaza substitution does not require any ribose modification (Carroll, S., unpublished observation).

The physical basis for the improved recognition of the 7-deaza substitution by HCV RdRp is not clear but could be a result of changes in glycosidic bond angle or length, or changes in the electronic character of the purine base, or possibly suggestive of a direct interaction between a hydrophobic region of the RdRp active site and the 7-position of the purine base. A number of substituents at the 7-deaza position are tolerated by HCV RdRp, including 7-methyl (**11**), 7-carboxamide (sangivamycin analog, **12**), 7-cyano (toyocamycin analog, **13**) with little change in inhibitory potency in the enzyme assay. However, in general, substitutions at the 7-position give rise to cytotoxicity in the cell-based assay and are therefore not useful [40, 42]. A trend towards decreasing cytotoxicity was noted for the 7-halogen substituted series, 7-Br > 7-Cl > 7-F (compounds **14-16**). The 7-F-7deaza-2'-C-methyladenosine analog was found to be essentially non-cytotoxic by MTS assay in the replicon cells at 100 μM, but to have the most potent replicon EC_{50} measured for a nucleoside analog (EC_{50} = 0.07 μM) [40]. Further investigations of **16** are underway.

The 7-deaza-adenosine modification is beneficial not just in terms of target enzyme potency but also in terms of stabilization of the analog to unwanted modification by adenosine-metabolizing enzymes. Most notably, the 7-deaza-modification essentially eliminates the ability of adenosine deaminase (ADA) to convert the 2'-C-methyladenosine to the corresponding inosine analog which is inactive in the either the replicon or enzyme assays. Under conditions where 2'-C-methyl adenosine is quantitatively converted to 2'-C-methylinosine in *in vitro* ADA-catalyzed reactions, 2'-C-methyl-7-deazaadenosine is left intact [40]. The lack of conversion of the 7-deaza-adenosine analog is related to the mechanism of catalysis by ADA, wherein the 7-nitrogen is protonated to aid in the delocalization of charge that occurs during formation of a tetrahedral intermediate that results in displacement of the 6-amino group by hydroxyl anion [43].

PNP catalyzes the phosphorolysis of the purine glycosidic bond, resulting in removal of the modified ribose, and allowing for subsequent metabolism of the ribose. The 2'-C-methyl substitution itself reduces the ability of PNP to catalyze glycosidic bond breaking [39]. However, the 7-deaza modification further stabilizes the glycosidic bond to PNP-catalyzed breakdown, further increasing the metabolic stability of 7-deaza-substituted nucleoside analogs [40]. These results demonstrating increased stability to adenosine-metabolizing enzymes *in vitro* correlate well with increased pharmacokinetic stability *in vivo* as discussed below.

MECHANISM OF CHAIN TERMINATION BY 2'-C-METHYL NUCLEOTIDES

The physical mechanism of the inhibition of RdRp activity by 2'-C-methyl-modified nucleotides appears to involve disruption of the growth of the RNA strand after incorporation of the modified nucleotide. Typically nucleoside analogs used as antiviral therapeutics act as chain terminators due to the absence of the 3'-hydroxyl group that functions as the nucleophile during extension of the DNA chain by incorporation of a nucleotide. Thus, analogs such as AZT, 3TC, ddC, d4T and the active forms of didanosine, and abacavir are incorporated onto the 3'-end of a growing primer strand but are unable to support further elongation of the growing strand. The family of 2'-C-methyl modified nucleotides appears to act *via* a similar mechanism despite the fact that the 3'-hydroxyl is still present in the 2'-C-methyl nucleotides. The 2'-C-methyl nucleotides are therefore non-obligate chain terminators.

The evidence supporting chain termination as the mechanism of inhibition by 2'-C-methyl nucleotides comes from analysis of polyacrylamide gel electrophoresis – based nucleotide incorporation experiments. Hairpin RNAs formed from synthetic oligoribonucleosides have been found to serve as efficient templates for RNA synthesis catalyzed by HCV RdRp [44]. An example of the designed structure of an RNA hairpin template is shown in Fig. (**3**), which has been labeled with ^{32}P at its 5'-end *via* polynucleotide kinase. The RNA template and products resulting from the incorporation of nucleotides or nucleotide analogs are then separated *via* gel electrophoresis and visualized *via* PhosphorImaging. In the case of this RNA template sequence, the first nucleotide to be incorporated would be an adenosine or adenosine analog, which would be followed by incorporation of a uridine or uridine analog, as shown in the control reactions on the left side of the gel. HCV RdRp is capable of incorporating 3'-deoxy-adenosine monophosphate, but is incapable of extending the 3'-deoxy-adenosine monophosphate once it has been incorporated, as expected for this obligate chain terminating analog, as shown in lanes 6 and 7. HCV RdRp is also capable of incorporating 7-deaza-adenosine monophosphate (tubercidin monophosphate) into this RNA hairpin, and is also capable of extending the incorporated 7-deaza-adenosine by addition of the next nucleotide, as shown in lanes 8 and 9. However even though HCV RdRp can incorporate either 2'-C-methyladenosine or 2'-C-methyl-7-deaza-adenosine into the RNA template, the enzyme is incapable of adding the next nucleotide onto the end of the analog-terminated template. Thus the 2'-C-methyl nucleoside analogs act as chain terminators.

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