Resistance to excision determines efficiency of inhibition of hepatitis C virus RNA-dependent RNA polymerase by nucleotide analogs including sofosbuvir

Brian Villalba#, Jiawen Li§, and Kenneth A. Johnson*

From the 1Institutes for Cell and Molecular Biology, University of Texas at Austin, Austin, Tx. 78712

Running Title: Mechanism of NS5B inhibition by nucleotide analogs

#§Present Addresses: #MoMa Therapeutics, 215 First St., Suite150 Cambridge, Ma. 02142; §Singular Genomics Systems, Inc., 10931 N. Torrey Pines Rd., La Jolla, Ca. 92037

*To whom correspondence should be addressed: Dr. Kenneth A. Johnson, Department of Molecular Biosciences, Institute for Cell and Molecular Biology, University of Texas at Austin, #4800. 2500 Speedway, MBB 3.122 Austin, TX 78712 Phone: (512) 471-0434, Fax: (512) 471-0435, E-mail: kajohnson@mail.utexas.edu

Keywords: hepatitis C virus (HCV), nonstructural protein 5B (NS5B), inhibition mechanism, enzyme kinetics, RNA-dependent RNA polymerase, viral polymerase, mericitabine, sofosbuvir, antiviral drug, chain terminator

Abstract

Nonstructural protein 5B (NS5B) is the viral RNA-dependent RNA polymerase that catalyzes the replication of the hepatitis C virus genome. It is a major target for antiviral drugs, including nucleotide analogs (NAs) such as the prodrugs mericitabine and sofosbuvir, which get metabolized to 2'-fluoro-2'-C-methylcytidine-5'-triphosphate and 2'-fluoro-2'-C-methyluridine-5'-triphosphate, respectively. These analogs act as chain terminators after they are incorporated during viral RNA synthesis. Recently, it has been shown that NS5B can efficiently remove chain terminators by a nucleotide-mediated excision reaction that rescues RNA synthesis. In this study, we used transient-state kinetics to study the efficiency of NS5B inhibition by five NAs. We show that NS5B readily incorporates CTP analogs into a growing primer, but that these analogs are also efficiently excised. In contrast, although UMP analogs were more slowly incorporated, UMP excision was also slow and inefficient, and modifications to the 2'C of the UTP ribose ring further decreased excision rates to an undetectable level. Taken together, these results suggest that the greater clinical effectiveness of the UMP analog sofosbuvir is largely due to it being intractable to nucleotide-mediated excision compared with similar NAs such as mericitabine.

Introduction

The Hepatitis C Virus (HCV) infects an estimated 3% of the world’s population with approximately 2.4 million people in the United States currently infected (1,2). Chronic HCV infections lead to hepatic fibrosis, cirrhosis, and hepatocellular carcinoma (3). HCV is a plus-sense single-stranded RNA virus containing a 9.6 kb genome which encodes a single poly-protein (4). This poly-protein consists of three structural proteins and seven nonstructural proteins including NS5B, the RNA-dependent RNA polymerase responsible for replicating the viral genome. NS5B catalyzes de novo initiation of RNA synthesis, which is inefficient in vitro but is followed by a transition to an efficient and fast processive elongation phase (5,6).

NS5B is the target for many antiviral therapies including nucleoside analogs, (7-9). These analogs work by mimicking the natural nucleotides and are incorporated during processive replication of the viral genome (10). Once incorporated, the analogs act as chain terminators by hindering polymerization of the next incoming base to prevent further elongation of the nascent RNA strand (11). These analogs include the prodrugs Mericitabine and Sofosbuvir, which get metabolized to 2'-fluoro-2'-C-methylcytidine-5'-triphosphate and 2'-fluoro-2'-C-methyluridine-5'-triphosphate, respectively (7,12,13).
Recent advancements have afforded formation and isolation of a processive elongation complex of NS5B with RNA in vitro (5). Transient-state kinetic methods have uncovered an efficient ATP-mediated excision reaction, which may play a role in maintaining fidelity during genome replication (14). Interestingly, ATP-mediated excision can also efficiently remove chain terminators thereby rescuing RNA synthesis. This mechanism was also observed in AZT-resistant variants of HIV RT and is thought to be the main cause of AZT resistance (15-18). However, wild-type NS5B has been shown to be approximately 50-fold more efficient than AZT-resistant forms of HIV RT in removing chain terminators via ATP-mediated excision (14). This raises questions regarding how any chain terminating nucleoside analog could be effective in treating HCV infections.

In this study we use transient-state kinetic methods to measure the efficiencies of incorporation and excision of several nucleoside analogs (Fig. 1). We show that CTP and CTP analogs are efficiently incorporated but are also readily excised. Furthermore, although there is a large discrimination against incorporation of the 2'-modified UTP analog compared to unmodified UTP, ATP-dependent excision and pyrophosphorylisis of UTP and its analog are very inefficient.

Results
Incorporation of NTP and NTP analogs
Analysis of the kinetics of nucleotide incorporation under single turnover conditions afforded the apparent equilibrium dissociation constants for ground-state binding (K$_{d, app}$) and the rate constants for the maximum rate of incorporation (k$_{pol}$) for CTP and UTP. Although the rates of incorporation of CTP and UTP were sufficiently fast to require the use of rapid-quench-flow methods, the rates of incorporation of the analogs were slow, so the kinetics could be measured using hand-mixing methods. The reactions were measured under single turnover conditions because the NS5B/9-nt/20-nt elongation complex pauses after the first incorporation due to the absence of the next complementary NTP (nucleoside triphosphate) and the dissociation of RNA from the enzyme is exceedingly slow (5). These measurements afforded the specificity constant (k$_{cal}$/K$_{m}$ = k$_{pol}$/K$_{d, app}$). The discrimination against incorporation of each analog was defined by the ratio of the specificity constant for the canonical nucleotide divided by that for the corresponding analog. The results for incorporation of CTP and CTP analogs (Fig. 2A-D) and UTP and the UTP analog (Fig. 3A-B) were fit based on Scheme 1 using KinTek Explorer software (Austin, TX). The results are summarized in Table 2. The kinetic parameters for incorporation of CTP (k$_{pol}$ = 21 ± 3 s$^{-1}$, K$_{d, app}$ = 46 ± 9 µM, k$_{pol}$/K$_{d, app}$ = 0.46 ± 0.1 µM$^{-1}$s$^{-1}$) are in agreement with previously reported results under similar conditions (5). The K$_{d, app}$ for 2'C-Me-2'F-CTP (59 ± 14 µM), 2'C-Me-CTP (61 ± 11 µM), and 4’-azido-CTP (23 ± 7 µM) were similar to that of CTP. However, the maximum rate constant for incorporation for each of the three CTP analogs (1.9 ± 0.4 s$^{-1}$, 1.0 ± 0.2 s$^{-1}$, and 1.5 ± 0.4 s$^{-1}$) was significantly lower than for unmodified CTP.

The k$_{pol}$ and K$_{d, app}$ for UTP incorporation were 33 ± 5 s$^{-1}$ and 320 ± 60 µM, respectively. giving a k$_{pol}$/K$_{d, app}$ of 0.1 ± 0.02 µM$^{-1}$s$^{-1}$. Similar to the case with the CTP analogs, the K$_{d, app}$ for incorporation of 2'C-Me-2'F-UTP (410 ± 50 µM) was comparable to that for UTP. The maximum rate constant for incorporation of the UTP analog was 0.3 ± 0.03 s$^{-1}$, roughly 100-fold lower than the rate constant for incorporation of UTP so that k$_{pol}$/K$_{d, app}$ = 0.0007 ± 0.0001 µM$^{-1}$s$^{-1}$. The enzyme discriminates against incorporation of 2'C-Me-2'F-UTP by a factor 140 ± 45 relative to UTP. These results differ from previously published results for incorporation of the UTP analog (19) where the reported K$_{d, app}$ = 113 µM, k$_{pol}$ = 0.67 s$^{-1}$, and k$_{pol}$/K$_{d, app}$ = 0.0059 µM$^{-1}$s$^{-1}$, were approximately 8-fold more efficient compared to our measurements. This may be due to a difference in the strain used, as well as a difference in the sequence of the RNA template. Nevertheless, both results reflect a large discrimination against incorporation of the analog.

Our results reveal that the modifications of the ribose ring do not influence the apparent binding affinity of the analog; rather, discrimination against these analogs is driven by reduced values of k$_{pol}$. All nucleoside analogs tested were shown to be effective chain terminators except for 4’-azido-CTP (data not shown). Therefore, the rest of this study focuses on the 2'C-modified nucleoside analogs.
Pyrophosphorolysis of Incorporated NMPs and 2′ C-Modified NMP analogs

To examine the kinetics of excision by pyrophosphorolysis, the NS5B elongation complex was generated in the presence of either the unmodified nucleotide or the 2′C-modified analog to generate an NS5B/10-nt/20-nt enzyme/primer/template complex. We then mixed the complex with 0—2 mM sodium pyrophosphate supplemented with a 1:1 ratio of MgCl$_2$:pyrophosphate. The results were fit using Scheme 1, where $K_3$ is the apparent equilibrium dissociation constant for pyrophosphate, $k_2$ is the rate constant for pyrophosphorolysis, and $k_{\phi}/K_1$ is the specificity constant for pyrophosphorolysis. The results are summarized in Table 3.

The rate constant for pyrophosphorolysis of CMP-terminated RNA ($k_2$) was measured to be $0.3 \pm 0.03 \text{ s}^{-1}$ and $K_1$ was determined to be $410 \pm 50 \text{ M}^{-1}$ resulting in a specificity constant of $(7.3 \pm 1) \times 10^{-4} \text{ M}^{-1} \text{s}^{-1}$ (Fig. 4A). These results are well within range of previously reported results (14). The rate constants for pyrophosphorolysis for 2′C-Me-CMP (Fig. 4B) and 2′C-Me-2′F-CMP (Fig. 4C) are 0.4 ± 0.04 s$^{-1}$ and 0.3 ± 0.03 s$^{-1}$, respectively. These rate constants are comparable to the rate constant for pyrophosphorolysis of CMP. There is a larger difference in the binding of pyrophosphate during excision of the analogs ($820 \pm 100 \text{ M}$ for 2′C-Me-CMP and $1600 \pm 200 \text{ M}$ for 2′C-Me-2′F-CMP) when compared to CMP (410 ± 50 M). These data indicate that binding of pyrophosphate, instead of the rate constant for excision accounts for the difference in the specificity constant for pyrophosphorolysis.

According to our results, NS5B catalyzes pyrophosphorolysis of CMP more efficiently than 2′C-Me-CMP ($k_2/K_1$ for CMP is $(7.3 \pm 1) \times 10^{-4}$ M$^{-1}$s$^{-1}$ versus $(4.6 \pm 0.8) \times 10^{-4}$ M$^{-1}$s$^{-1}$). 2′C-Me-2′F-CMP is most resistant to pyrophosphorolysis ($k_2/K_1 = (1.8 \pm 0.2) \times 10^{-4}$ M$^{-1}$s$^{-1}$) compared to CMP and 2′C-Me-CMP. It is important to note that the apparent equilibrium constant for pyrophosphate binding will include a term for translocation of the RNA from the nucleotide binding site to the primer binding site. A more favorable equilibrium constant for translocation will reduce the apparent affinity for pyrophosphate binding. Thus, 2′C-modified nucleoside analogs may shift the translocation to move the 3′ nucleotide of the RNA away from the reaction center.

Our data demonstrate that the 2′F modification further increases resistance to excision via pyrophosphorolysis. We also noted that the amplitude of the observed reaction depended on pyrophosphate concentration indicating that the reaction came to equilibrium such that the net reaction was linked to concentration of added pyrophosphate. This could be due to slow release or rebinding of the nucleoside triphosphate (NTP) product of the reaction. We fit the data according to Scheme 1 by allowing the rebinding and reincorporation of the NTP to afford estimates of $k_2$ and the apparent equilibrium constant for NTP rebinding ($K_1$) to yield the specificity constant, $K_k/k_2$. The measured specificity constant for NTP reincorporation is nearly identical to the results from the nucleotide incorporation data. For this reason, we globally fit data for the kinetics of incorporation and pyrophosphorolysis to obtain the values for rate constant listed in Table 3.

UMP undergoes pyrophosphorolysis significantly less efficiently (Fig. 6A) compared to CMP and the CMP analogs. The rate constant for pyrophosphorolysis was measured to be $0.007 \pm 0.002 \text{ s}^{-1}$, and the apparent affinity for pyrophosphate binding was $1700 \pm 200 \text{ M}$. This slower rate constant and weaker binding results in a specificity constant of $(0.041 \pm 0.02) \times 10^{-4}$ M$^{-1}$ s$^{-1}$, ~178-fold less than the $k_2/K_1$ for pyrophosphorolysis of CMP. As with the pyrophosphorolysis of CMP and CMP analogs, an amplitude dependence was observed with increasing concentration of pyrophosphate due to the reincorporation of UTP. Under these conditions, $k_2$ and $K_1$ are almost identical to the results measured during the UTP incorporation reaction, and were globally fit with data for kinetics of incorporation and pyrophosphorolysis to obtain the values for rate constants listed in Table 3.

We attempted to measure the pyrophosphorolysis of 2′C-Me-2′F-UMP (Fig. 7A). No pyrophosphorolysis was observed at any pyrophosphate concentration over the time course measured. To set an upper limit on the rate constant for pyrophosphorolysis, we estimate that within the limits of detection <1% of the input substrate reacted at the highest concentration of
Mechanism of NS5B inhibition by nucleotide analogs

pyrophosphate. Accordingly, we set an upper limit of \( k_s K_s \leq 0.0001 \times 10^2 \mu M^{-1}s^{-1} \).

Together, these results indicate that efficiency of pyrophosphorolysis is dependent on the base, and modifications to the 2'-carbon on the ribose ring further increase resistance to pyrophosphorolysis.

**ATP-Mediated Excision of NMP and 2'C-Modified NMP analogs**

We measured the efficiency of ATP-mediated excision by generating the NS5B/10-nt/20-nt elongation complex as described in the methods section. We then incubated the complex with 0-8 mM ATP supplemented with a 1:1 molar ratio of MgCl2:ATP. The results were fit using Scheme 2 where \( k_5 \) is the rate constant for ATP-mediated excision, \( I/K_s \) is the apparent equilibrium dissociation constant for binding ATP during excision, and \( K_d k_5 \) is the specificity constant for ATP-mediated excision. We observed an ATP concentration dependence on the amplitude of the excision reaction, implying a reversible link between ATP binding and the equilibrium endpoint of the reaction. We account for the amplitude dependence by including the reverse reaction. That is, the dinucleoside tetraphosphate (Ap\( N \)) product generated during the excision reaction can be reincorporated into the primer strand so the excision reaction comes to equilibrium. The rate constant for Ap\( N \) reincorporation is defined by \( k_5 \), the apparent equilibrium dissociation constant is \( K_d \), and \( k_s K_5 \) defines the specificity constant for reincorporation. Although the apparent \( K_d \) and maximum rate of the reaction of Ap\( N \) are not defined, the data provide reasonable limits on the estimate of the specificity constant for reaction of Ap\( N \), \( k_s K_d \). The results are summarized in Table 4.

The kinetic parameters for ATP-mediated excision of CMP (Fig. 5A) were comparable to previously published results (\( k_5 = 0.043 \pm 0.006 \text{ s}^{-1} \), \( I/K_5 = 2.8 \pm 0.4 \text{ mM} \), \( K_d k_5 = (1.5 \pm 0.3) \times 10^{-5} \mu M^{-1} \text{s}^{-1} \)). The incorporated CTP analogs were excised at slightly slower rates (\( k_5 = 0.034 \pm 0.002 \text{ s}^{-1} \) and \( 0.024 \pm 0.002 \text{ s}^{-1} \) for 2'C-Me-2'C-MP and 2'C-Me-2'F-MP, respectively) when compared to excision of CMP. NS5B has a slightly higher apparent affinity for ATP during excision of 2'C-Me-2'C-MP (1.6 \pm 0.67 mM, Fig. 5B), but the apparent affinity remains relatively unchanged during excision of 2'C-Me-2'F-CMP (2.1 \pm 0.1 mM, Fig. 5B). After incorporation, CTP and CTP analogs are readily excised via this mechanism. Therefore, the resulting specificity constants indicate that 2'C-Me-CMP is ~1.3-fold more efficiently excised, \( K_d k_5 = (2.0 \pm 0.1) \times 10^{-5} \mu M^{-1} \text{s}^{-1} \), while 2'C-Me-2'F-CMP is ~1.3-fold less efficiently excised, \( K_d k_5 = (1.2 \pm 0.1) \times 10^{-5} \mu M^{-1} \text{s}^{-1} \), compared to CMP.

The specificity constant for the reverse of ATP-mediated excision (reaction of Ap\( C \); \( k_s K_6 = 0.38 \pm 0.2 \mu M^{-1} \text{s}^{-1} \)) is similar to values previously reported (5). As with the reincorporation of the Ap\( C \), estimates for the specificity constant for reincorporation of the Ap\( C \)-analogs can be obtained from the data. The specificity constant for the reverse of the ATP-mediated excision for 2'C-Me-CMP (\( k_s K_6 = 0.47 \pm 0.2 \mu M^{-1} \text{s}^{-1} \)) is similar to that of Ap\( C \). For 2'C-Me-2'F-CMP, the efficiency of reincorporation of the dinucleoside is over 2-fold higher compared to Ap\( C \) (\( k_s K_6 = 0.92 \pm 0.3 \mu M^{-1} \text{s}^{-1} \)). This indicates that the modifications play a role in increasing the efficiency of reincorporation of the dinucleoside tetraphosphate after ATP-mediated excision.

ATP-mediated excision of UMP is much less efficient than the excision of CMP and its analogs (Fig. 6B). At the highest ATP concentrations, only ~20% of the input NS5B/10-nt/20-nt complex was excised by the end of the time course and saturation could not be reached. Therefore, we were only able to estimate the lower limit on the rate constant for excision to calculate the specificity constant from these data. The specificity constant was determined from the ATP concentration-dependence of the rate of excision, (0.05 \pm 0.02) \times 10^{-6} \mu M^{-1} \text{s}^{-1}. This result indicates that, as is the case with pyrophosphorolysis, the efficiency of ATP-mediated excision is greatly reduced with UMP compared to CMP. Moreover, upon attempting to measure ATP-mediated excision of 2'C-Me-2'F-UMP, no excision was observed at any concentration of ATP (Fig. 7B). We set upper limits on the rate constant for ATP-mediated excision of 2'C-Me-2'F-UMP by NS5B by estimating a lower limit for detectable product of <1% turnover by the end of the time course. This lower limit gives an estimated \( K_d k_5 \leq 0.004 \times 10^{-6} \mu M^{-1} \text{s}^{-1}. \) Therefore, not only does the uracil base lead to resistance to ATP-mediated excision, but also the 2'C modifications further reduce the efficiency of excision. By evading ATP-dependent
excision, Sofosbuvir provides an effective treatment, while Mericitabine fails because it is rapidly removed by excision.

Sequence Dependence of Excision of Nucleoside Analogs

Previous studies on various polymerases have demonstrated nucleotide incorporation and excision can be influenced by local sequence context (20-23). To survey whether certain sequences are more or less susceptible to incorporation of nucleotide analogues and ATP-mediated excision, a processive elongation assay was performed in the presence of the analogs and a high concentration of ATP. The elongation complex was assembled as indicated in the methods using a 45-nt template to generate an NS5B/9-nt/45-nt complex. The 45-nt template was designed to contain multiple opportunities for the analogs to be incorporated. The elongation complex was then mixed with all four NTP along with a nucleoside analog. The reactions were performed using 50 µM or 3 mM ATP. The results were fit using Scheme 3. According to the template sequence, we expected to see incorporation of the CTP analogs and chain termination at the 11-, 29-, 36-, 40-, and 44-nt position of the primer strand. Both CTP analogs were efficiently incorporated at each position except for the 29-nt position. This may indicate that there is some sequence dependence for incorporation. Nevertheless, the chain-terminators were able to inhibit full extension of the primer strand (Figs. 8 and 9). By monitoring the reduction in band intensity over time, excision was observed at the 11-nt position of the primer for both CTP analogs at low ATP (Figs. 8A and 9A) and high ATP (Figs. 8B and 9B). The rate constant for excision at the 11-nt position was 0.088 ± 0.01 s⁻¹ and 0.028 ± 0.01 s⁻¹ for 2'C-Me-CTP and 2'C-Me-2'-F-CTP, respectively. Little excision was observed at the 36-, 40-, and 44-nt position. This may indicated that efficiency of the excision of the CTP analogs may depend somewhat on local sequence.

For 2'C-Me-2'-F-UTP, we expect to observe incorporation at the 10-, 15-, 17-, 18-, 22-, 26-, 28-, 33-, and 43-nt positions of the primer strand. We observed incorporation at each position except for the 43-nt position. Regardless of the ATP concentration, after incorporation of the analog the curves stay completely flat indicating no excision was occurring (Fig. 10). Fitting these results using Scheme 3 yields rate constants for excision that are essentially zero. This demonstrates that the resistance to ATP-mediated excision of the 2'-modified UMP analog appears to be universal and is not dependent on the context of the sequence.

Discussion

In this study, we set out to understand the kinetic basis for NS5B inhibition by nucleoside analogs, and to assess whether they are able to resist ATP-dependent excision. By isolating an active NS5B elongation complex, we were able to determine the kinetics of incorporation for CTP, UTP, and their respective analogs. NS5B discriminates against incorporation of CTP analogs between 6-fold to 27-fold with respect to incorporation of the correct substrate. However, there is a 140-fold discrimination against incorporation of the 2'C-Me-2'-F-UTP analog when compared to incorporation of UTP. This result is ~3-fold higher than previously reported (140-fold vs. 45-fold, (19)). This difference could possibly be explained by the difference in the strains used (Con1 strain vs. BK strain). When comparing the differences in discrimination, one would conclude that the 2'C-modified CTP analogs would be more effective inhibitors. However, 4'-azido-CTP, which showed the lowest discrimination, allowed for slow elongation on top of the azido-CMP and is therefore not an effective chain-terminator. Also, it is known from clinical trials that Mericitabine, the prodrug which becomes metabolized into 2'C-Me-2'-F-CTP, is less effective at treating HCV infections when compared to Sofosbuvir (10,19). Our data suggest that the effectiveness of the nucleoside analog is not determined by their kinetics of incorporation. Rather, the effectiveness of the nucleoside analog is driven by the ability to resist ATP-dependent excision after incorporation.

Pyrophosphorolysis or ATP-mediated excision reactions could be used to remove chain terminators. In our studies, pyrophosphorolysis occurs more efficiently on the natural CMP base than in the 2'C-Me-CMP analog. The 2'-fluoro modification further decreases the efficiency of pyrophosphorolysis ~2.5-fold indicating that the 2'C-modifications play a role in mitigating the reversal of analog incorporation. However, our data
Mechanism of NS5B inhibition by nucleotide analogs

Demonstrate that the efficiency of pyrophosphorolysis is dependent on the incorporated base. Pyrophosphorolysis of UMP is ~245-fold less efficient than that of CMP. Furthermore, our data show that the addition of the 2'C-Me-2'-F modification further lowered the efficiency of pyrophosphorolysis to a level undetectable in our assays. Therefore, our data support a model in which the efficiency of pyrophosphorolysis is determined not only by the base, but also by the modifications at the 2'-carbon of the ribose ring.

Pyrophosphorolysis and ATP-mediated excision require that 3' end of the RNA primer be in the active site. Following polymerization, the RNA duplex translocates to move the 3' end out of the active site to allow binding of the next NTP. Thus translocation will reduce the observed rate of reaction with either ATP or pyrophosphate. It is likely that pyrophosphate (or ATP) will bind only to the un-translocated state. The $K_{d,app}$ for binding pyrophosphate would be attenuated by the equilibrium constant for translocation. Thus, it is likely that the identity of the base and modifications may alter rate and the equilibrium constant for translocation. Differences in the translocation equilibrium may explain differences in reactivity in comparing CMP and UMP.

The efficiency of pyrophosphorolysis is between 15-50 higher than ATP-mediated excision for CMP and CMP analogs, and 91-fold higher for UMP. However, the average physiological concentrations of pyrophosphate and ATP are approximately 3.5 µM and 3 mM, respectively (24,25). Therefore, ATP-mediated excision will occur at a faster rate than pyrophosphorolysis under physiological conditions (Fig. 11).

ATP-mediated excision by HIV RT plays a significant role in the emergence of AZT-resistance (16,26,27). Our work has demonstrated that wild type NS5B catalyzes the ATP-mediated excision of CMP 25-fold more efficiently than AZT-resistant variants of HIV RT (14,16). Addition of the 2'-C-Me-Me modification slightly increased the efficiency of ATP-mediated excision compared to CMP, but the addition of the 2'C-Me-2'-F modification mitigated the efficiency of ATP-mediated excision. However, ATP-mediated excision of the analogs is significantly higher when compared to the rates of ATP-mediated excision of AZT by resistant mutants of HIV RT (34-fold higher for 2'C-Me-CMP and 20-fold higher for 2'C-Me-2'-F-CMP (16). In contrast, ATP-mediated excision of UMP by NS5B is incredibly inefficient. Our results demonstrated that the efficiency is approximately tenfold lower that AZT excision by HIV RT. The further addition of the 2'C modifications further mitigated the efficiency of ATP-mediated excision to a level undetectable above background. Estimates of the upper limit of the rate constant for ATP-mediated excision of the UMP analog indicate that the efficiency is at least 130-fold lower than AZT excision by resistant HIV RT variants.

Previous studies have demonstrated that a serine to threonine mutation at the 282 position confers resistance to Sofosbuvir (28,29). However, this mutation has not been observed in any clinical isolates. A low frequency of Sofosbuvir-resistant mutations emerged during clinical trials (L159F and V321A), which may have contributed to the treatment failing to mitigate the viral infection (30). These mutants required an intensified treatment to achieve sustained virologic response. Our attempts to study the effect of these mutants have been limited due to an inability to isolate NS5B variants that can make it through the inefficient de novo initiation to generate an active elongation complex. More work is needed to optimize conditions in order to determine if the observed resistance is due to an increase in discrimination against incorporation or an increase in the efficiency of excision reactions.

Our work demonstrates that although 2'C-modified CTP analogs are more efficiently incorporated compared to the UTP analog, the UTP analog is a better inhibitor because of its resistance to ATP-dependent excision. Once the UTP analog is incorporated, it exhibits a half-life of greater than 24 hours before ATP-mediated excision occurs (Fig. 12). The CTP analogs, however, have a half-life of 30-50 seconds (Fig. 12), and are, therefore, less efficient inhibitors under physiological conditions. This resistance is attributed to both the base itself as well as the additional 2'C-Me-2'-F modifications.

Materials and Methods

Nucleic acids and Chemicals

CTP- and UTP-analogs used in this study were generously provided in triphosphate form by Janssen Pharmaceutical, Inc. and Gilead Sciences,
Expression and Purification of NS5BΔA21

N-terminal hexa-His-NS5BΔA21 (Con1 strain, GT1b with the C-terminal 21-amino acid membrane anchor domain deleted) was cloned into a pC1(ts, ind+) vector (31) under control of a rightward promoter from bacteriophage lambda controlled by a chemically- and temperature-inducible lambda repressor encoded on the plasmid. Plasmids were transformed into NEB Turbo E. coli cells and cultured in Terrific Broth at 30°C overnight. One liter of media was inoculated with the cultures grown overnight at an A$_{600}$ of 0.1 and grown at 30°C until an A$_{600}$ of 4 was reached. Expression was induced by the addition of nalidixic acid to a final concentration of 50 µg/mL, and the temperature was raised to 37°C. After 16 hours, cells were collected, and the pellets were stored at -80°C. Cells were resuspended in lysis buffer (50 mM HEPES, pH 7.5, 300 mM NaCl, 2 mM DTT, 20% (v/v) Glycerol, 0.1% (w/v) Octyl β-D-glucopyranoside) and EDTA solutions were purchased from Ambion (Austin, TX). Octyl β-D-glucopyranoside and DTT were purchased from GoldBio (St. Louis, MO).

Assembly and Isolation of Elongation Complex

A reaction containing 12 µM NS5B, radiolabeled 20 µM pGG, 20 µM RNA Template was incubated at 30°C for 1.5 hours to form an elongation complex of NS5B with a nine nucleotide primer and a 20 or 45 nucleotide template (NS5B/9-nt/20-nt or 45-nt). For the 20-nt CG template 50 µM ATP and UTP were added. For the 20-nt UA and 45-nt templates 50 µM ATP and GTP were added. Reactions were carried out in buffer containing 40 mM Tris-HCl, pH 7.0, 40 mM NaCl, 5 mM DTT, and 2 mM MgCl$_2$. The elongation complex was isolated by centrifuging at 16,000 rpm for 5 minutes using a benchtop centrifuge. The supernatant was discarded, and the pellet was washed twice with wash buffer (40 mM Tris-HCl, pH 7.0, 20 mM NaCl, 5 mM DTT, and 2 mM MgCl$_2$) to remove contaminants. The pellet was resuspended in a buffer containing 40 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM DTT, and 2 mM MgCl$_2$.

Assembly and Isolation of NS5B/10-nt/20-nt Elongation Complex

The reaction described above was performed to generate the NS5B/9-nt/20-nt elongation complex followed by the addition of 20 µM next correct NTP or 100 µM of nucleoside analog. The reaction was incubated at 30°C for 20 seconds for NTP incorporation or 5 minutes for nucleoside analog incorporation to generate the NS5B/10-nt/20-nt elongation complex. The elongation complex was pelleted, washed twice, and resuspended as described above.

Measurement of Incorporation of Nucleoside Triphosphate and Nucleoside Analogs

The elongation complex was assembled, washed, and resuspended in a buffer containing 40 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM DTT, and 2 mM MgCl$_2$. To measure the kinetics of NTP incorporation, resuspended elongation complex

Mechanism of NS5B inhibition by nucleotide analogs

respectively. The RNA templates, pGG dimer and onGG dimer were purchased from Dharmaco, Inc. (Chicago, IL). The sequences of the RNA templates used are shown in Table 1. The onGG dimer was labeled with [γ-$^{32}$P]-ATP (PerkinElmer) using T4 polynucleotide kinase from NEB (Ipswich, MA) according to the protocol recommended by NEB. The reaction was stopped by incubating at 95°C for 3 minutes. The radiolabeled pGG was diluted 10-fold with cold pGG to make a radiolabeled stock solution of a known concentration. Ribonucleoside triphosphates were purchased from Promega (Madison, WI). Tris-HCl buffers, NaCl, MgCl$_2$, and EDTA solutions were purchased from Ambion (Austin, TX). Octyl β-D-glucopyranoside and 20% (v/v) Glycerol, and 0.1% (w/v) Octyl β-D-glucopyranoside. The concentration was determined by measuring the absorbance at 280 nm using an extinction coefficient of 170,850 cm$^{-1}$M$^{-1}$ (5). The protein was stored at -80°C until use.

Expression and Purification of NS5BA21

N-terminal hexa-His-NS5BΔA21 (Con1 strain, GT1b with the C-terminal 21-amino acid membrane anchor domain deleted) was cloned into a pC1(ts, ind+) vector (31) under control of a rightward promoter from bacteriophage lambda controlled by a chemically- and temperature-inducible lambda repressor encoded on the plasmid. Plasmids were transformed into NEB Turbo E. coli cells and cultured in Terrific Broth at 30°C overnight. One liter of media was inoculated with the cultures grown overnight at an A$_{600}$ of 0.1 and grown at 30°C until an A$_{600}$ of 4 was reached. Expression was induced by the addition of nalidixic acid to a final concentration of 50 µg/mL, and the temperature was raised to 37°C. After 16 hours, cells were collected, and the pellets were stored at -80°C. Cells were resuspended in lysis buffer (50 mM HEPES, pH 7.5, 300 mM NaCl, 2 mM DTT, 20% (v/v) Glycerol, 0.1% (w/v) Octyl β-D-glucopyranoside, 400 mM Imidazole) over 10 column volumes. Peak fractions were pooled, concentrated, and dialyzed into storage buffer (30 mM Tris-HCl, pH 7.5, 400 mM NaCl, 5 mM DTT, 20% (v/v) Glycerol, and 0.1% (w/v) Octyl β-D-glucopyranoside). The concentration was determined by measuring the absorbance at 280 nm using an extinction coefficient of 170,850 cm$^{-1}$M$^{-1}$ (5). The protein was stored at -80°C until use.

Assembly and Isolation of Elongation Complex

A reaction containing 12 µM NS5B, radiolabeled 20 µM pGG, 20 µM RNA Template was incubated at 30°C for 1.5 hours to form an elongation complex of NS5B with a nine nucleotide primer and a 20 or 45 nucleotide template (NS5B/9-nt/20-nt or 45-nt). For the 20-nt CG template 50 µM ATP and UTP were added. For the 20-nt UA and 45-nt templates 50 µM ATP and GTP were added. Reactions were carried out in buffer containing 40 mM Tris-HCl, pH 7.0, 40 mM NaCl, 5 mM DTT, and 2 mM MgCl$_2$. The elongation complex was isolated by centrifuging at 16,000 rpm for 5 minutes using a benchtop centrifuge. The supernatant was discarded, and the pellet was washed twice with wash buffer (40 mM Tris-HCl, pH 7.0, 20 mM NaCl, 5 mM DTT, and 2 mM MgCl$_2$) to remove contaminants. The pellet was resuspended in a buffer containing 40 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM DTT, and 2 mM MgCl$_2$.

Assembly and Isolation of NS5B/10-nt/20-nt Elongation Complex

The reaction described above was performed to generate the NS5B/9-nt/20-nt elongation complex followed by the addition of 20 µM next correct NTP or 100 µM of nucleoside analog. The reaction was incubated at 30°C for 20 seconds for NTP incorporation or 5 minutes for nucleoside analog incorporation to generate the NS5B/10-nt/20-nt elongation complex. The elongation complex was pelleted, washed twice, and resuspended as described above.

Measurement of Incorporation of Nucleoside Triphosphate and Nucleoside Analogs

The elongation complex was assembled, washed, and resuspended in a buffer containing 40 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM DTT, and 2 mM MgCl$_2$. To measure the kinetics of NTP incorporation, resuspended elongation complex
was rapidly mixed with an equal volume of solution containing NTP in the same buffer using an RQF-3 rapid quench-flow instrument (KinTek Corp., Austin, TX). Reactions were quenched in a solution containing 50 mM EDTA and collected and stored in solution containing 50 mM EDTA, 90% formamide, 0.1% bromophenol blue, and 0.1% xylene cyanol FF. To measure the kinetics of nucleoside analog incorporation, the elongation complex was mixed by hand with an equal volume of solution containing nucleoside analog in the same buffer. At given timepoints aliquots of the reaction solution were transferred to a quench solution containing 50 mM EDTA, 90% formamide, 0.1% bromophenol blue, and 0.1% xylene cyanol FF. The samples were denatured by incubating at 95°C for 5 minutes and loaded onto 16% denaturing polyacrylamide gel containing 7M urea. Electrophoresis was performed at 100 W and 50°C using BioRad Sequi-Gen GT System. Gels were dried at 80°C for 1 hour using BioRad Model 583 Gel Dryer and exposed to a storage phosphor screen. Screen was imaged using GE Healthcare Typhoon 9400 scanner. Band intensities were quantified using ImageQuant (GE Healthcare). Product formation was calculated as the fractional intensity of each product band relative to the total intensity of all bands in the given lane.

**Pyrophosphorolysis and ATP-Mediated Excision**

The NS5B/10-nt/20-nt elongation complex was generated, pelleted, and resuspended in buffer as described above. The elongation complex was mixed with an equal volume of solution containing sodium pyrophosphate or ATP and incubated at 30°C. For a given timepoint an aliquot was removed from the reaction and quenched in solution containing 50 mM EDTA, 90% formamide, 0.1% bromophenol blue, and 0.1% xylene cyanol FF. The samples were denatured by incubating at 95°C for 5 minutes and loaded onto a 16% denaturing polyacrylamide gel containing 7M urea. Electrophoresis, drying, exposing, and quantification were conducted as described in previous sections. The concentrations of each nt position over time were plotted versus time in Microsoft Excel.

**Data analysis**

The kinetics of incorporation, pyrophosphorolysis, and ATP-mediated excision were fit using KinTek Explorer (KinTek Corp., Austin, TX) to determine the rate constants of the respective reactions as described previously (32,33). The kinetics of incorporation were determined by fitting using Scheme 1. In order to fit the data to determine the $K_{d,\text{app}}$ for NTP binding the rate constant for NTP or nucleotide analog binding ($k_i$) was assumed to be close to diffusion limited and locked at 100 µM$^{-1}$s$^{-1}$, and the rate constant for dissociation ($k_i$) was allowed to vary during fitting. The apparent equilibrium dissociation constant was then calculated by dividing the rate constant for dissociation by the rate constant for binding ($K_{d,\text{app}} = 1/K_i = k_{i,d}/k_i$). The maximum rate constant for polymerization ($k_{pol}$) afforded definition of the rate constant $k_2$. Because the amount of pyrophosphate produced during the reaction is low relative to nucleotide, the reaction is largely irreversible; therefore, $k_{2,d}$ was locked at 0. Pyrophosphate release is fast and not rate-limiting, so the $k_3$ is locked at 100 s$^{-1}$. The specificity constant ($k_{cat}/K_m = k_{pol}/K_{d,\text{app}}$) was determined by dividing the rate constant for polymerization by the $K_{d,\text{app}}$ for nucleotide binding ($k_{i,d}/K_{d,\text{app}} = K_i/k_2$).

The kinetics of pyrophosphorolysis were determined by fitting using the reverse reactions.
Mechanism of NS5B inhibition by nucleotide analogs

shown in Scheme 1. Pyrophosphorolysis reactions were globally fit including incorporation reactions in order to account for the amplitude dependence observed. The rate constant for pyrophosphate binding ($k_{-3}$) was assumed to be diffusion limited and locked at 100 $\mu$M$^{-1}$s$^{-1}$. The rate constants for pyrophosphate dissociation ($k_3$) and pyrophosphorolysis ($k_{-2}$) were allowed to vary during fitting. The $K_{d,app}$ for pyrophosphate was determined by dividing the rate constant for pyrophosphate release by the rate constant for binding ($K_{d,app} = K_3 = k_3/k_{-3}$). The specificity constant for pyrophosphorolysis ($k_{cat}/K_m$) was determined by dividing the rate constant for pyrophosphorolysis by the $K_{d,app}$ for pyrophosphate binding ($k_{cat}/K_m = K_{d,app}$).

The kinetics of ATP-mediated excision were determined by fitting using Scheme 2. To determine the apparent equilibrium dissociation constant for ATP ($K_{d,app}$), the rate constant for ATP binding ($k_4$) was assumed to be diffusion limited and locked at 100 $\mu$M$^{-1}$s$^{-1}$. The rate constant for ATP dissociation ($k_{-4}$) was allowed to vary during the fitting process. The rate constant for the dissociation of ATP was then divided by the rate constant for binding to determine the $K_{d,app}$ ($1/K_4 = k_{-4}/k_4$). The maximum rate constant for ATP-mediated excision was determined by allowing $k_5$ to vary during the fitting process. The specificity constant was determined by multiplying $k_5$ by $1/K_{d,app}$ ($k_{cat}/K_m = K_{d,app}$). The rates for the reverse of ATP-mediated excision ($k_3$) and Ap$_4$N release ($k_6$) were linked at a constant ratio during fitting. Due to the low concentration of Ap$_4$N, the rate constant for Ap$_4$N rebinding ($k_{-6}$) was assumed to be slow and locked at 1 $\mu$M$^{-1}$s$^{-1}$.

For the processive elongation in the presence of nucleotide analog, the concentration of each chain-terminated primer position was plotted versus time in KinTek Explorer. The curves for each primer position were fit by simulation using the processive elongation model in Scheme 3. This model accounts for NTP incorporation (E.R$_n$ $\rightarrow$ E.R$_{n+1}$), chain termination due to nucleotide analog incorporation (E.R$_n$ $\rightarrow$ E.R$_x$), and possible ATP-mediated excision leading to RNA synthesis rescue (E.R$_x$ $\rightarrow$ E.R$_n$.Ap$_4$NA $\rightarrow$ E.R$_n$).

Data Availability

The authors confirm that the data supporting the findings of this study are available within the article.
Acknowledgements: This work was supported by grants from NIAID (1R01AI110577 to KAJ) and the Welch Foundation (F-1604 to KAJ). 2’C-Me-CTP, 2’C-Me-2’-F-CTP, and 4’-azido-CTP were provided by Janssen Pharmaceutical, Inc. 2’C-Me-2’-F-UTP was provided by Gilead Sciences.

Conflict of interest: KAJ is president of KinTek Corporation, which provided the RQF-3 rapid quench-flow instrument and KinTek Explorer software used in this study.
Mechanism of NS5B inhibition by nucleotide analogs

References

1. Hofmeister, M. G., Rosenthal, E. M., Barker, L. K., Rosenber, E. S., Barranco, M. A., Hall, E. W., Edlin, B. R., Mermin, J., Ward, J. W., and Ryerson, A. B. (2019) Estimating Prevalence of Hepatitis C Virus Infection in the United States, 2013-2016. Hepatology 69, 1020-1031

2. Shepard, C. W., Finelli, L., and Alter, M. J. (2005) Global epidemiology of hepatitis C virus infection. Lancet Infect Dis 5, 558-567

3. Chak, E., Talal, A. H., Sherman, K. E., Schiff, E. R., and Saab, S. (2011) Hepatitis C virus infection in USA: an estimate of true prevalence. Liver Int.

4. Ashfaq, U. A., Javed, T., Rehman, S., Nawaz, Z., and Riazuddin, S. (2011) An overview of HCV molecular biology, replication and immune responses. Virol J. 8

5. Jin, Z., Leveque, V., Ma, H., Johnson, K. A., and Klumpp, K. (2012) Assembly, Purification, and Pre-steady-state Kinetic Analysis of Active RNA-dependent RNA Polymerase Elongation Complex. J. Biol. Chem. 287, 10674-10683

6. Li, J., and Johnson, K. A. (2016) Thumb Site 2 Inhibitors of Hepatitis C Viral RNA-dependent RNA Polymerase Allosterically Block the Transition from Initiation to Elongation. J. Biol. Chem. 291, 10067-10077

7. Sofia, M. J., Bao, D., Chang, W., Du, J., Nagarathnam, D., Rachakonda, S., Reddy, P. G., Ross, B. S., Wang, P., Zhang, H.-R., Bansal, S., Espiritu, C., Keilman, M., Lam, A. M., Micolochick Steuer, H. M., Niu, C., Otto, M. J., and Furman, P. A. (2010) Discovery of a β-D-2'-Deoxy-2'-α-fluoro-2'-β-C-methyluridine Nucleotide Prodrug (PSI-7977) for the Treatment of Hepatitis C Virus. J. Med. Chem. 53, 7202-7218

8. Sofia, M. J., Chang, W., Furman, P. A., Mosley, R. T., and Ross, B. S. (2012) Nucleoside, Nucleotide, and Non-Nucleoside Inhibitors of Hepatitis C Virus NS5B RNA-Dependent RNA-Polymerase. J. Med. Chem. 55, 2481-2531

9. Götte, M., and Feld, J. J. (2016) Direct-acting antiviral agents for hepatitis C: structural and mechanistic insights. Nat. Rev. Gastroenterol. Hepatol. 13, 338–351

10. Eltahla, A. A., Luciani, F., White, P. A., Lloyd, A. R., and Bull, R. A. (2015) Inhibitors of the Hepatitis C Virus Polymerase; Mode of Action and Resistance. Viruses 7, 5206-5224

11. Appleby, T. C., Perry, J. K., Murakami, E., Barauskas, O., Feng, J., Aesop, C., Fox III, D., Wetmore, D. R., McGrath, M. E., Ray, A. S., Sofia, M. J., Swaminathan, S., and Edwards, T. E. (2015) Structural basis for RNA replication by the hepatitis C virus polymerase. Science 347, 771-775

12. Murakami, E., Bao, H., Ramesh, M., McBrayer, T. R., Whitaker, T., Micolochick Steuer, H. M., Schinazi, R. F., Stuyver, L. J., Obikhod, A., Otto, M. J., and Furman, P. A. (2007) Mechanism of Activation of β-D-2-Deoxy-2-Fluoro-2-C-Methylcytidine and Inhibition of Hepatitis C Virus NS5B RNA Polymerase. Antimicrob. Agents and Chemother. 51, 503-509

13. German, P., Mathias, A., Brainard, D., and Kearney, B. P. (2016) Clinical Pharmacokinetics and Pharmacodynamics of Ledipasvir/Sofosbuvir, a Fixed-Dose Combination Tablet for the Treatment of Hepatitis C. Clin. Pharmacokinet. 55, 1337-1351

14. Jin, Z., Leveque, V., Ma, H., Johnson, K. A., and Klumpp, K. (2013) NTP-mediated nucleotide excision activity of hepatitis c virus RNA-dependent RNA polymerase. Proc. Natl. Acad. Sci. U.S.A. 110, E348-E357
Boyer, P. L., Sarafianos, S. G., Arnold, E., and Hughes, S. H. (2002) Nucleoside Analog Resistance Caused by Insertions in the Fingers of Human Immunodeficiency Virus Type 1 Reverse Transcriptase involves ATP-Mediated Excision. *J. Virol.* **76**, 9143-9151

Meyer, P. R., Matsuura, S. E., and Scott, W. A. (1999) A Mechanism of AZT Resistance: An Increase in Nucleotide-Dependent Primer Unblocking by Mutant HIV-1 Reverse Transcriptase. *Mol. Cell* **4**, 35-43

Sluis-Cremer, N., Sheen, C.-W., Zelina, S., Argoti Torres, P. S., Parikh, U. M., and Mellors, J. W. (2007) Molecular Mechanism by Which the K70E Mutation in Human Immunodeficiency Virus Type 1 Reverse Transcriptase Confers Resistance to Nucleoside Reverse Transcriptase Inhibitors. *Antimicrob. Agents and Chemother.* **51**, 48-53

Tu, X., Dad, K., Han, Q., Bauman, J. D., Clark Jr., A. D., Hou, X., Frenkel, Y. V., Gaffney, B. L., Jones, R. A., Boyer, P. L., Hughes, S. H., Sarafianos, S. G., and Arnold, E. (2010) Structural basis of HIV-1 resistance to AZT by excision. *Nat. Struct. Mol. Biol.* **17**, 1202-1209

Fung, A., Jin, Z., Dyatkina, N., Wang, G., Beigelman, L., and Deval, J. (2014) Efficiency of Incorporation and Chain Termination Determines the Inhibition Potency of 2'-Modified Nucleotide Analogs against Hepatitis C Virus Polymerase. *Antimicrob. Agents and Chemother.* **58**, 3636-3645

Bloom, L. B., Otto, M. R., Eritja, R., Reha-Krantz, L. J., Goodman, M. F., and Beechem, J. (1994) Pre-Steady-State Kinetic Analysis of Sequence-Dependent Nucleotide Excision by the 3'-Exonuclease Activity of Bacteriophage T4 DNA Polymerase. *Biochemistry* **33**, 7576-7586

Mendelman, L. V., Boosalis, M. S., Petruska, J., and Goodman, M. F. (1989) Nearest Neighbor Influences on DNA Polymerase Insertion Fidelity. *J. Biol. Chem.* **264**, 14415-14423

Pless, R. C., and Bessman, M. J. (1983) Influence of Local Nucleotide Sequence on Substitution of 2-Aminopurine for Adenine during Deoxyribonucleic Acid Synthesis in Vitro. *Biochemistry* **22**, 4905-4915

Petruska, J., and Goodman, M. F. (1985) Influence of Neighboring Bases on DNA Polymerase Insertion and Proofreading Fidelity. *J. Biol. Chem.* **260**, 7533-7539

Ryan, L. M., Kozin, F., and McCarty, D. J. (1979) Quantification of Human Plasma Inorganic Pyrophosphate. *Arthritis Rheum.* **22**, 886-891

Traut, T. W. (1994) Physiological concentrations of purines and pyrimidines. *Mol. Cell. Biochem.* **140**, 1-22

Meyer, P. R., Matsuura, S. E., So, A. G., and scott, W. A. (1998) Unblocking of chain-terminated primer by HIV-1 reverse transcriptase through a nucleotide-dependent mechanism. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 13471-13476

Ray, A. S., Murakami, E., Basavapathruni, A., Vaccaro, J. A., Ulrich, D., Chu, C. K., Schinazi, R. F., and Anderson, K. S. (2003) Probing the Molecular Mechanisms of AZT Drug Resistance Mediated by HIV-1 Reverse Transcriptase Using a Transient Kinetic Analysis. *Biochemistry* **42**, 8831-8841

Migliaccio, G., Tomassini, J. E., Carroll, S. S., Tomei, L., Altamura, S., Bhat, B., Bartholomew, L., Bosserman, M. R., Ceccacci, A., Colwell, L. F., Cortese, R., de Francesco, R., Eldrup, A. B., Getty, K. L., Hou, X. S., LaFemina, R. L., Ludmerer, S. W., MacCoss, M., McMasters, D. R., Stahlhut, M., W., Olsen, D. B., Hazuda, D. J., and Flores, O. A. (2003) Characterization of Resistance to Non-obligate Chain-terminating
Ribonucleoside Analogs that Inhibit Hepatitis C Virus Replication in Vitro. J. Biol. Chem. 278, 49164-49170

29. Svarovskaia, E. S., Dvoyr-Sobol, H., Parkin, N., Hebner, C., Gontcharova, V., Martin, R., Ouyang, W., Han, B., Xu, S., Ku, K., Chiu, S., Gane, E., Jacobson, I. M., Nelson, D. R., Lawitz, E., Wyles, D. L., Bekele, N., Brainard, D., Symonds, W. T., McHutchinson, J. G., Miller, M. D., and Mo, H. (2014) Infrequent Development of Resistance in Genotype 1–6 Hepatitis C Virus–Infected Subjects Treated With Sofosbuvir in Phase 2 and 3 Clinical Trials. Clin. Infect. Dis. 59, 1666-1674

30. Svarovskaia, E. S., Gane, E., Dvoyr-Sobol, H., Martin, R., Doehle, B., Hedskog, C., Jacobson, I. M., Nelson, D. R., Lawitz, E., Brainard, D., McHutchinson, J. G., Miller, M. D., and Mo, H. (2016) L159F and V321A Sofosbuvir-Associated Hepatitis C Virus NS5B Substitutions. J. Infect. Dis. 213, 1240-1247

31. Brandis, J. W., and Johnson, K. A. (2009) High-cell Density Shake-flask Expression and Rapid Purification of the Large Fragment of Thermus aquaticus DNA Polymerase I Using a New Chemically and Temperature Inducible Expression Plasmid in Escherichia coli. Protein Expr. Pur., 120-127

32. Johnson, K. A., Simpson, Z. B., and Blom, T. (2009) Global Kinetic Explorer: A new computer program for dynamic simulation and fitting of kinetic data. Anal. Biochem. 387, 20-29

33. Johnson, K. A., Simpson, Z. B., and Blom, T. (2009) FitSpace Explorer: An algorithm to evaluate multidimensional parameter space in fitting kinetic data. Anal. Biochem. 387, 30-41
FOOTNOTES
The abbreviations used are: HCV, Hepatitis C virus; NS5B, non-structural protein 5B; nt, nucleotide; Ap4N, Adenine dinucleoside tetraphosphate; NTP, nucleoside triphosphate.
**Mechanism of NS5B inhibition by nucleotide analogs**

**Scheme 1.** Minimal model for incorporation and pyrophosphorolysis of nucleotide triphosphate. $E$ represents enzyme and $R_n$ represent an RNA primer $n$ residues in length.

\[
E \cdot R_9 + NTP \xleftrightarrow{K_1} E \cdot R_9 \cdot NTP \xrightarrow{k_2} E \cdot R_{10} \cdot PP_i \xleftarrow{k_3} E \cdot R_{10} + PP_i
\]

**Scheme 2.** Minimal model for ATP-Mediated Excision of incorporated nucleoside monophosphate

\[
E \cdot R_{10} + ATP \xrightarrow{K_4} E \cdot R_{10} \cdot ATP \xleftarrow{k_5} E \cdot R_9 \cdot Ap_4N \xrightarrow{k_6} E \cdot R_9 + Ap_4N
\]

**Scheme 3:** Minimal model for processive elongation in the presence of nucleotide analog. The above model is used to test for sequence dependence of ATP-mediated excision after NS5B chain termination. The various species are defined in the inset box.

**Table 1:** RNA Templates. The underlined base indicates the last template position before the reaction pauses during elongation complex assembly. The base in red indicates the templating base for the next incorporation.

| 20-nt  | 3'-CCUAUAUUAAGCAUAUCUAA-5' |
|--------|-----------------------------|
| 20-nt UA | 3'-CCUCUCUCUCAGAAUAUCUAA-5' |
| 45-nt  | 3'-CCUCUCUCUCAGUCUCAAUCAUCUACAGUUCACGCGUUCGCAGU-5' |
Table 2. HCV NS5B Nucleoside and Nucleoside Analog Incorporation Parameters

| Nucleotide | $K_{d,app}$ (µM) | $k_{pol}$ (s$^{-1}$) | $k_{pol}/K_{d,app}$ (µM$^{-1}$s$^{-1}$) | Discrimination |
|------------|-----------------|---------------------|-----------------------------------|----------------|
| CTP:G      |                 |                     |                                   |                |
| CTP        | 26 ± 3          | 10 ± 0.8            | 0.38 ± 0.05                       | -              |
| 2’C-Me-CTP | 48 ± 7          | 1.0 ± 0.1           | 0.021 ± 0.004                     | 18 ± 4         |
| 2’C-Me-2’-F-CTP | 63 ± 11   | 1.9 ± 0.3           | 0.030 ± 0.007                     | 13 ± 3         |
| UTP:A      |                 |                     |                                   |                |
| UTP        | 320 ± 60        | 33 ± 5              | 0.10 ± 0.02                       | -              |
| 2’C-Me-2’-F-UTP | 410 ± 50 | 0.3 ± 0.03          | 0.00073 ± 0.0001                  | 140 ± 45       |

Table 2. Summary of kinetic parameters for incorporation of nucleoside and nucleoside analog triphosphates. The rate constants were derived by fitting the data using Scheme 1. Standard errors were derived by nonlinear regression in globally fitting the data (32). Discrimination is determined using the equation ($k_{pol}/K_{d,app}$)$_{NTP}$/$k_{pol}/K_{d,app}$$_{Inhibitor}$.

Table 3. HCV NS5B Nucleoside and Nucleoside Analog Pyrophosphorolysis Parameters

| Nucleotide | $1/K_1$ (µM) | $k_2$ (s$^{-1}$) | $K_1k_2$ (µM$^{-1}$s$^{-1}$) | $k_2$ (s$^{-1}$) | $K_3$ (µM) | $k_2/K_3 \times 10^6$ (µM$^{-1}$s$^{-1}$) |
|------------|--------------|-----------------|-----------------------------|-----------------|-----------|----------------------------------------|
| CTP        | 21 ± 1       | 46 ± 4          | 0.46 ± 0.05                 | 0.3 ± 0.03      | 410 ± 50  | 730 ± 100                              |
| 2’C-Me-CTP | 1.7 ± 0.2    | 54 ± 7          | 0.032 ± 0.006               | 0.4 ± 0.04      | 820 ± 100 | 460 ± 83                               |
| 2’C-Me-2’-F-CTP | 1.8 ± 0.2 | 56 ± 7          | 0.031 ± 0.006               | 0.3 ± 0.03      | 1600 ± 200 | 180 ± 24                               |
| UTP        | 28 ± 5       | 310 ± 70        | 0.09 ± 0.02                 | 0.007 ± 0.002   | 1700 ± 200 | 4.1 ± 1                                |
| 2’C-Me-2’-F-UTP | *410  | *0.3            | *0.00073                    | -               | -         | ≤ 0.01                                 |

* Rate constants for 2’C-Me-2’-F-UTP incorporation are from Table 2

Table 3. Summary of kinetic parameters for pyrophosphorolysis of incorporated nucleosides and nucleoside analogs. The rate constants were derived by fitting the data sets using Scheme 1 as described in the text. Standard errors were derived by nonlinear regression in globally fitting the data (32). Confidence contour analysis supported the use of standard error estimates derived from nonlinear regression.
Table 4. HCV NS5B Nucleoside and Nucleoside Analog ATP-Mediated Excision

| Nucleotide       | $1/K_i$ ($\mu$M) | $k_s$ (s$^{-1}$) | $K_a k_s \times 10^4$ ($\mu$M$^{-1}$s$^{-1}$) | $k_a/K_a$ ($\mu$M$^{-1}$s$^{-1}$) |
|------------------|-----------------|----------------|---------------------------------------------|---------------------------------|
| CMP              | 2800 ± 400      | 0.043 ± 0.006  | 15 ± 3                                      | 0.38 ± 0.2                      |
| 2'C-Me-CMP       | 1600 ± 67       | 0.034 ± 0.002  | 20 ± 1                                      | 0.47 ± 0.2                      |
| 2'C-Me-2'-F-CMP  | 2100 ± 100      | 0.024 ± 0.002  | 12 ± 1                                      | 0.92 ± 0.3                      |
| UMP              | -               | -              | 0.05 ± 0.02                                 | -                              |
| 2'C-Me-2'-F-UMP  | -               | -              | ≤ 0.004                                     | -                              |

Table 4. Summary of kinetic parameters for ATP-mediated excision of incorporated nucleosides and nucleoside analogs. The rate constants were derived by fitting the data sets using Scheme 2 as described in the text. Standard errors were derived by nonlinear regression in globally fitting the data (32). The individual rates for the reverse reactions are not defined by the data and therefore only the specificity constant ($k_a/K_a$) is able to be determined.

Figure 1: Structure of nucleoside analogs- A) the structure of the produgs Mericitabine and Sofosbuvir are shown. B) the structure of the four nucleoside analogs are shown provided by Janssen Pharmaceutical,
Inc. (cytidine analogs) and Gilead Sciences (uridine analog). Analogs were provided with 5'-triphosphate.

**Figure 2: Incorporation of CTP and CTP analogs during elongation** - The time course for incorporation of A) CTP (2, 5, 15, 50, 100, and 200µM), B) 2’C-Me-CTP (1, 2.5, 5, 10, 50, and 100µM), C) 2’C-Me-2’-F-CTP (1, 2.5, 5, 10, 50, and 100µM) and D) 4’-azido-CTP (1, 2.5, 5, 10, 50, and 100µM) were collected using rapid quenched-flow. The data were fit using KinTek Explorer using Scheme 1. The solid line represents the best fit generated during data fitting. The results from the fit are summarized on in Table 2.
**Figure 3: Incorporation of UTP and 2’-C-Me-2’-F-UTP.** The above plots show the incorporation of A) UTP (10, 25, 100, 200, and 400 µM) and B) 2’C-Me-2’-F-UTP (12.3, 37, 111, 333, and 1000 µM). UTP incorporation was measured using rapid quenched-flow, and incorporation of 2’C-Me-2’-F-UTP was measured using hand quench methods. The data were fit using Scheme 1 using KinTek Explorer and the solid line shows the best fit generated during data fitting. Results are summarized in Table 2.

**Figure 4: Pyrophosphorolysis of CMP and 2’-C Modified CTP analogues.** The above plots show the pyrophosphorolysis A) CMP (31.25, 62.5, 125, 500, and 1000 µM), B) 2’C-Me-CMP (15.6, 31.3, 62.5, 125, 250, and 500 µM) and C) 2’C-Me-2’-F-CMP (15.6, 31.3, 62.5, 125, 250, and 500 µM). The data were fit using Scheme 2 using KinTek Explorer. Solid lines represent best fit resulting from the fitting process. Results are summarized in Table 3.
**Figure 5: ATP-Mediated Excision of 2’C Modified CTP analogues** - These plots show the ATP-Mediated Excision of A) CMP, B) 2’C-Me-CMP, and C) 2’C-Me-2’F-CMP. ATP concentrations used were 0.25, 0.5, 1, 2, 4, and 8 mM. The data were fit using Scheme 3 using KinTek Explorer. The solid lines show the best fit generated during the fitting process. Results are summarized in Table 3.

**Figure 6: Excision of UTP** - The above plots show UMP excision by A) pyrophosphorolysis (250, 500, 1000, and 2000 μM), and B) ATP-Mediated Excision (1, 2, 4, and 8 mM). The results were fit to Schemes 2 and 3, respectively, using KinTek Explorer. The solid lines represent the best fit generated during fitting. Results are summarized in Table 3.
Figure 7: Excision of 2′C-Me-2′-F-UTP – A) A representative 16% denaturing PAGE separation of UMP pyrophosphorolysis is shown. Bands below the 9-nt band appear due to pyrophosphorolysis occurring at that position. All bands below 10 nucleotides were summed during analysis to obtain the correct concentration of the loss of the 10-nt substrate due to pyrophosphorolysis. B-C) The above show the results for B) pyrophosphorolysis of 2′-C-Me-2′-F-UMP and C) ATP-Mediated 2′-C-Me-2′-F-UMP Excision. Reactions were separated on a 16% PAGE gel containing 8 M urea. Line on (B) indicates where gels are spliced together. No excision products were detectable during the observed time course. Addition of the next correct nucleotide did not result in extension of the primer strand to an 11 nt product (CTP control; first lane in B. and C.) indicating effective chain termination.
Mechanism of NS5B inhibition by nucleotide analogs

Figure 8: Sequence dependence of ATP-mediated 2'C-Me-CTP excision by NS5B. The processive elongation of a primer in the presence of 500 µM 2'C-Me-CTP and 50 µM ATP (A) or 3 mM ATP (B) is shown. Final concentrations for CTP, GTP, and UTP were 50 µM in each experiment. The color of the curves corresponds to nucleotide position in the primer sequence above. The solid lines represent the best fit generated during fitting using Scheme 3.
Mechanism of NS5B inhibition by nucleotide analogs

Figure 9: Sequence dependence of ATP-mediated of 2’C-Me-2’F-CTP by NS5B. Shown is the processive elongation of a primer in the presence of 500 µM 2’C-Me-2’F-CTP and 50 µM ATP (A) or 3 mM ATP (B). Final concentrations for CTP, GTP, and UTP were 50 µM in each experiment. The color of the curves corresponds to nucleotide position in the primer sequence above. The solid lines represent the best fit generated during fitting using Scheme 3.

Primer: 5’-GGAGAGAAGUCAGAUGUAAAGUAGAUGUAGUGCCAGAUG-3’
Template: 3’-CCUCUCUCAGUCUCAAUUAUCUACGUACCAGUCCG-5’
Figure 10: Sequence dependence of ATP-mediated of 2′C-Me-2′-F-UTP by NS5B. The above plots show the processive elongation of a primer in the presence of 1 mM 2′C-Me-2′-F-UTP with 50 µM ATP (A) or 3 mM ATP (B). CTP, GTP, and UTP were present to a final concentration of 50 µM of each nucleoside triphosphate for both experiments. The color of each curve corresponds to the position in the primer sequence above. The solid lines represent the best fit generated during fitting using Scheme 3.
Figure 11: Comparison of rates of excision reactions under physiological conditions - The above chart compares the rates of pyrophosphorolysis and ATP-mediated excision for CMP, 2'-modified CMP analogs, and UMP at physiological concentrations. Rates were calculated using 3.5 μM for pyrophosphate and 3 mM for ATP. The numbers above the bar show the calculated rates x 10^{-2} s^{-1}. Error bars define the standard error.
**Figure 12: Half-life of CMP, UMP, and nucleotide analogs.** The chart shows the half-life of each incorporated nucleotide and analog due to ATP-mediated excision. Half-life was calculated using the equation \( t_{1/2} = \frac{\ln(2)}{k} \) where \( k \) is the rate of ATP-mediated excision at physiological concentrations of ATP (Fig. 11).