Nucleotide Substrate Specificity of Hepatitis C Analogs for Human Mitochondrial RNA Polymerase

Maryam Ehteshami[a], Longhu Zhou[a], Sheida Amiralaei[a], Jadd R. Shelton[a], Jong Hyun Cho[a], Hongwang Zhang[a], Hao Li, Xiao Lu[a], Tugba Ozturk[a], Richard Stanton[a], Franck Amblard[a], Tamara R. McBrayer[b], Steven J. Coats[b], and Raymond F. Schinazi[a #]

[a] Center for AIDS Research, Laboratory of Biochemical Pharmacology, Department of Pediatrics, Emory University School of Medicine, Atlanta, GA, USA

[b] Cocrystal Pharma, Inc., Tucker, GA, USA

Running title: Nucleoside analogs as substrates of POLRMT

[#] - Address correspondence to Raymond F. Schinazi, Emory University, HSRB, Room E-420, 1760 Haygood Drive, Atlanta, GA 30322, USA.
Email: rschina@emory.edu
Tel: +1-40-727-1414, Fax: +1-404-727-1330

ABSTRACT (75 words maximum)

Nucleoside analog inhibitors (NAI) are an important class of antiviral agents. Although highly effective, some NAI with anti-hepatitis C virus (HCV) activity can cause toxicity presumably due to off-target inhibition of host mitochondrial RNA polymerase (POLRMT). The *in vitro* nucleotide substrate specificity of POLRMT enzyme was studied in order to explore structure-

activity relationships that can facilitate the identification of non-toxic NAI. These finding have important implications for all anti-RNA virus NAI development.

TEXT

Nucleoside analog inhibitors (NAI) have been an important component of antiviral therapy for decades. 2'-Deoxyribonucleoside analogs are regularly used for treatment of viral infections such as human immunodeficiency virus (HIV), hepatitis B virus (HBV) and herpes simplex virus (HSV) (reviewed in (1, 2)). Upon cellular entry, NAIs must be phosphorylated by viral or cellular kinases into their active 5'-triphosphate (TP) form. NAI-TPs are substrates for the viral polymerase and their incorporation often results in chain-termination of the growing genome and subsequent inhibition of virus replication. Recently, sofosbuvir became the first ribonucleoside analog inhibitor to receive approval for treatment of hepatitis C virus (HCV), an RNA virus (3). Sofosbuvir is administered as a phoshoramidate prodrug in order to target the liver, improve cell permeability and bypass the first phosphorylation step by host cell kinases (4, 5). Despite the excellent safety profile of sofosbuvir, developments of several preceding anti-HCV ribonucleotide prodrugs were halted due to toxicity (reviewed in (6)). For example, phase II clinical trials with BMS-986094 (formerly known as INX-189) were terminated after reports of severe adverse events and one death (7-9). Although the exact mechanism of toxicity is not yet fully elucidated, it has been shown that treatment with NAI can sometimes lead to off-target inhibition of host polymerases. For example, off-target inhibition of mitochondrial DNA polymerase γ has been well documented for zidovudine triphosphate (AZT-TP), the first anti-HIV 2'-deoxyribonucleoside analogs to received FDA approval (10-13). Importantly, it was recently shown that ribonucleoside analog triphosphates could be substrates for recombinant human mitochondrial RNA polymerase (POLRMT) (14-16). Unlike the nuclear counterpart

RNA POL II, POLRMT lacks exonuclease activity and, therefore, incorporation of chain-terminating nucleotides was shown to be irreversible *in vitro* (14). Consistent with these observations, Arnold *et al.* reported that increased POLRMT incorporation of NAI-TPs, as well as high intracellular NAI-TP levels, correlated positively with reductions in mitochondrial RNA levels and cytotoxicity (14). Similarly, we recently showed that treatment of Huh-7 cells with BMS-986094, which generates high levels of 2'-*C*-methyl-GTP intracellularly, inhibited mitochondrial RNA transcription (17). Interestingly, treatment with a 2,6-diaminopurine nucleotide (DAPN) prodrug, an investigational anti-HCV agent that also generates 2'-*C*-methyl-GTP (18), did not have similar deleterious effects. Lower intracellular NAI-TP accumulation was proposed to account for the distinct cytotoxic profiles of these compounds (17).

Considering the importance of safety in developing novel ribonucleoside analogs as inhibitors of HCV and other RNA viruses, we explored the nucleotide substrate specificity of POLRMT. Here we report on the *in vitro* POLRMT incorporation profiles of over forty ribonucleoside analog 5'-triphosphates in order to shed light on structure-activity relationships that lead to the identification of non-toxic NAI. NAI-TPs that were not substrates for this enzyme were further examined for antiviral activity. Knowledge gained from this study has important implications not only for anti-HCV, but all anti-RNA virus antiviral NAI development.

Incorporation of nucleoside analogs by POLRMT. In order to examine the nucleoside substrate specificity of POLRMT, we evaluated the incorporation profile of 41 nucleosides with various chemical modifications on the ribose or base moiety (chemical structures summarized in Figure 1). As previously described (14, 17), 125 nM of purified POLRMT enzyme was

incubated with 500 nM of 5'-radiolabeled RNA/DNA primer/ template that allowed for the incorporation of A, C, G, or U nucleotide analogs at position +1, respectively. 100 μM of each nucleoside 5'-triphosphate analog was incubated with the POLRMT:RNA/DNA complex containing the appropriate DNA template for two hours at 30 °C. The amount of NAI-TP incorporation was normalized to the corresponding natural rNTP substrate (Figure 2). We determined that most modifications on the ribose ring of nucleotide analogs did not completely neutralize incorporation by POLRMT. As depicted in Figure 2a, addition of 2'-*O*-methyl, 2'-fluoro, or 2'-amino modifications did not consistently affect POLRMT incorporation for all nucleotides tested. Consistent with previous findings (14, 15), addition of a *C*-methyl group at the 2' position generally led to notable reductions in incorporation for C, G, and U analogs. Nucleotides harboring this modification have been examined for their antiviral activity against HCV (19, 20) and dengue virus (21). Interestingly, the effect of the 2'-*C*-methyl modification was especially pronounced for 2'-*C*-methyl-UTP whose incorporation was reduced to 10 % of natural UTP, suggesting that U analogs may be particularly vulnerable to chemical modifications with regards to POLRMT incorporation. This is in agreement with the observations that 2'-*C*-methyl-2'-F-UTP, the active metabolite of sofosbuvir, is an exceedingly poor substrate for PORLMT (Figure 2a). Under the conditions tested, we found 3'- or 2'-deoxynucleoside triphosphates were generally good substrates for POLRMT (with the exception of 3'-dCTP) (Figure 2b). However, the simultaneous removal of both hydroxyl groups (2',3'-ddNTPs) completely abrogated incorporation, suggesting that the presence of at least one OH group is essential for NAI-TP incorporation. Our data are consistent with previous observations that little discrimination exists against sugar-modified nucleotides (22). The observed promiscuity of POLRMT has implications for fidelity of this enzyme during mitochondrial RNA transcription.

Considering that 2'-deoxynucleoside triphosphate analogs were substrates for POLRMT, we next asked whether dNAI-TPs active against DNA viruses or retroviruses might also be substrates for POLRMT (see Figure 1b for chemical structures). As expected, DAPD-TP, DXG-TP, AZT-TP and ETV-TP were not incorporated by POLRMT. Surprisingly, we found 30% and 36% incorporation for 3TC-TP and GCV-TP, respectively at 100 µM of the NTP (Figure 2c). Although extensive literature exists on the impact of dNAI-TPs on mitochondrial DNA synthesis, it is worth noting that little information is available on the role of these compounds with regards to interference with mitochondrial RNA transcription in cells.

In our search for nucleoside analogs that are not substrates for POLRMT, but active against the viral RNA polymerase of HCV, we next investigated the incorporation profiles of nucleoside analog 5'-triphosphates harboring various base moiety modifications (Figure 1c). Of the nine compounds tested, we found that the majority of modifications at positions 4, 5, 6 and 8 of purines and pyrimidines did not significantly reduced incorporation by POLRMT (Figure 2c). Similarly, nucleoside analog triphosphates containing bulky modifications (such as 8-azido-ATP, 4-thio-UTP and 5-bromo-UTP) were readily incorporated by POLRMT. The combined presence of 2'-*C*-methyl and 2-fluoro modifications resulted poor POLRMT incorporation (12% of the natural ATP substrate). We have previously reported on the anti-HCV activity of this compound (23), where phosphoramidate prodrug of 2'-*C*-methyl-2-fluoro-ATP was found to inhibit HCV replicons with submicromolar activity.

The addition of NHOH chemical group on position 4 of pyrimidine ring (4-*N*-OH-CTP) was also observed to reduce incorporation by PORLMT to 50% of CTP (Figure 2c). Addition of 2'-*C*-

methyl group further reduced incorporation to 20% of CTP. Both 4-*N*-OH-CTP and 2'-*C*-methyl-4-*N*-OH-CTP demonstrated anti-HCV activity in cell culture (24, 25).

Incorporation of *N*1-methyl-GTP by POLRMT. We identified *N*1-methyl-GTP as a nucleoside analog 5'-triphosphate with minimal incorporation by POLRMT. Considering that the addition of the *N*1-methyl group on the base moiety reduced POLRMT incorporation to 10% of the natural GTP substrate (Figure 2c), *N*1-methyl-GTP was selected for further analysis with regards to incorporation by viral NS5B RNA polymerase. NS5B incorporation assays were performed as previously described (17). NS5B: RNA/RNA complexes were incubated with increasing concentrations of GTP or *N*1-methyl-GTP. Single nucleotide incorporation was observed over time and visualized on a denaturing polyacrylamide gel (Figure 3) where the 5'-radiolabeled 9mer primer was extended to a 10mer product. As expected, natural GTP substrate was rapidly incorporated with an apparent dissociation constant ($K_{d,app}$) value of 4.1 ± 1.6 μM (Figure 3a). Conversely, at least 125 μM of *N*1-methyl-GTP was required for 50 % incorporation by HCV NS5B enzyme ($K_{d,app}$ > 100 μM), suggesting compromised incorporation when compared to natural GTP (Figure 3b).

We next asked whether RNA extension could occur after *N*1-methyl-GTP incorporation by NS5B enzyme. NAI-TP incorporation was assessed in the presence of 10 μM CTP, ATP and UTP and increasing amounts of *N*1-methyl-GTP (Figure 4a). We found that as *N*1-methyl-GTP levels increased, an increase in full-length 20mer product was observed suggesting that nucleotide extension can occur following N1-methyl-GTP incorporation (Figure 4b, left panel). As expected full-length RNA synthesis was more readily observed with low levels of GTP (Figure 4b, right panel). Overall, these data suggested that although *N*1-methyl-GTP was a poor substrate for NS5B enzyme, its incorporation does not result in RNA chain-termination. We next

asked whether *N*1-methyl-GTP could inhibit RNA polymerization in the presence of competing GTP. We found that when 1 μM of GTP was present in addition to 10 μM of CTP, ATP and UTP, *N*1-methyl-GTP had no effect on full-length RNA synthesis (Figure 4c). This is in contrast to control compound 2'-*C*-methyl-GTP, which inhibited RNA synthesis with an IC_{50} value of 3.3 ± 0.5 μM (Figure 4c). Finally, we asked whether *N*1-methyl-GTP might inhibit viral genome synthesis in a manner that may not be detectable in our *in vitro* assay. To address this issue, we chemically synthesized a phosphoramidate prodrug of *N*1-methyl-GTP for cell culture testing (Scheme 1). We observed no inhibition when up to 200 μM of *N*1-methyl-G-phosphoramidate prodrug was incubated with HCV genotype 1b replicon cells (see (24) for experimental methods). Further experiments suggested that this prodrug is not efficiently triphosphorylated intracellularly (data not shown). Together, these data suggest that although *N*1-methyl-GTP may be a safe NAI-TP with regards to off-target incorporation by human POLRMT, it will likely not be effective as an anti-HCV agent. We also did not detect any antiviral activity against chikungunya, influenza A and respiratory syncytial virus when tested up to 100 μM. It remains to be determined whether this compound can be of interest as an antiviral agent for other RNA viruses.

In conclusion, in this report we examined the *in vitro* nucleotide substrate specificity of POLRMT. We found to our surprise that the POLRMT active site is relatively tolerant of incorporating most of the nucleoside analog 5'-triphosphates tested. Several anti-HCV NAI-TPs were identified to be poor substrates for POLRMT. In conclusion, the information on NAI-TP incorporation profile of POLRMT described herein sheds light on the biochemical properties of this enzyme active site and inform future ribonucleotide analog drug design for all RNA viruses.

Acknowledgements

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Abbreviations

HCV, hepatitis C virus; POLRMT, human mitochondrial RNA polymerase; NAI, Nucleoside analog inhibitors; TP, 5'-triphosphate.

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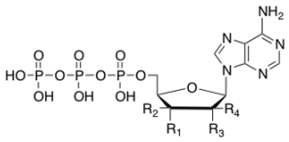
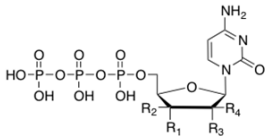
Figures

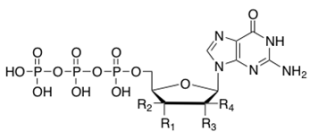
A



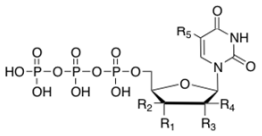
(1) ATP: R_1= OH, R_3= OH, R_2= R_4= H,
(2) 2'-dATP: R_1= OH, R_2= R_3= R_4= H
(3) 2', 3'-ddATP: R_1= R_2= R_3= R_4= H
(4) 3'-dATP: R_1= R_2= R_4= H, R_3= OH
(5) 2'-*O*-methyl-ATP: R_1= OH, R_2= R_4= H, R_3= OCH_3
(6) 2'-F-dATP: R_1= OH, R_2= R_4= H, R_3= F



(7) CTP: R_1= OH, R_2= R_4= H, R_3= OH
(8) 2'-dCTP: R_1= OH, R_2= R_3= R_4= H
(9) 2'-3'-ddCTP: R_1= R_2= R_3= R_4= H
(10) 2'-*O*-methyl-CTP: R_1= OH, R_2= R_4= H, R_3= OCH_3
(11) 2'-*C*-methyl-CTP: R_1= OH, R_2= H, R_3= OH, R_4= CH_3
(12) 2'-amino-CTP: R_1= OH, R_2= R_4= H, R_3= NH_2
(13) 3'dCTP: R1= R_2= R_4= H, R_3= OH
(14) 2'-F-dCTP: R_1= OH, R_2= R_4= H, R_3= F



(15) GTP: R_1= OH, R_2= R_4= H, R_3= OH
(16) 2'-dGTP: R_1= OH, R_2= R_3= R_4= H
(17) 2', 3'-ddGTP: R_1= R_2= R_3= R_4= H
(18) 2'-*C*-methyl-GTP: R_1= OH, R_2= H, R_3= OH, R_4= CH_3
(19) 3'-dGTP: R_1= R_2= R_4= H, R_3= OH
(20) 2'-*O*-methyl-GTP: R_1= OH, R_2= R_4= H, R_3= OCH_3
(21) 2'-F-dGTP: R_1= OH, R_2= R_4= H, R_3= F



(22) UTP: R_1= OH, R_2= R_4= R_5= H, R_3= OH
(23) 2'-dTTP: R_1= OH, R_2= R_3= R_4= H, R_5= CH_3
(24) 2', 3'-ddTTP: R_1= R_2= R_3= R_4= H, R_5= CH_3
(25) 2'-C-methyl-UTP: R_1= OH, R_2= R_5= H, R_3= OH, R_4= CH_3
(26) 2'-amino-UTP: R_1= OH, R_2= R_4= R_5= H, R_3= NH_2
(27) 2'-*C*-methyl-2'-F-dUTP: R_1= OH, R_2= R_5= H, R_3= F, R_4= CH_3
(28) 3'-dUTP: R_1= R_2= R_4= R_5= H, R_3= OH
(29) 2'-*O*-methyl-UTP: R_1= OH, R_2= R_4= R_5= H, R_3= OCH_3
(30) 2'-F-dUTP: R_1= OH, R_2= R_4=R_5= H, R_3= F

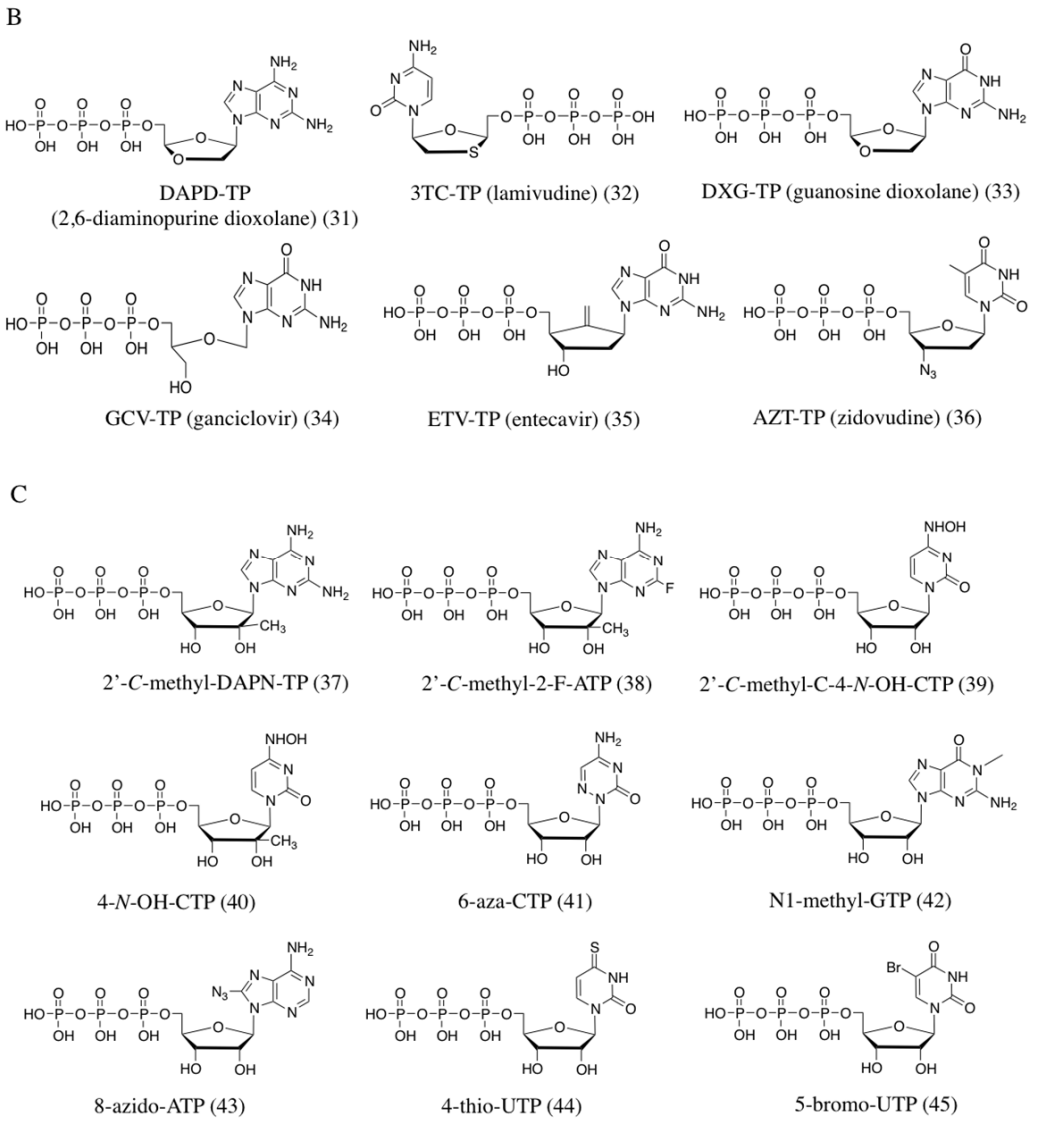
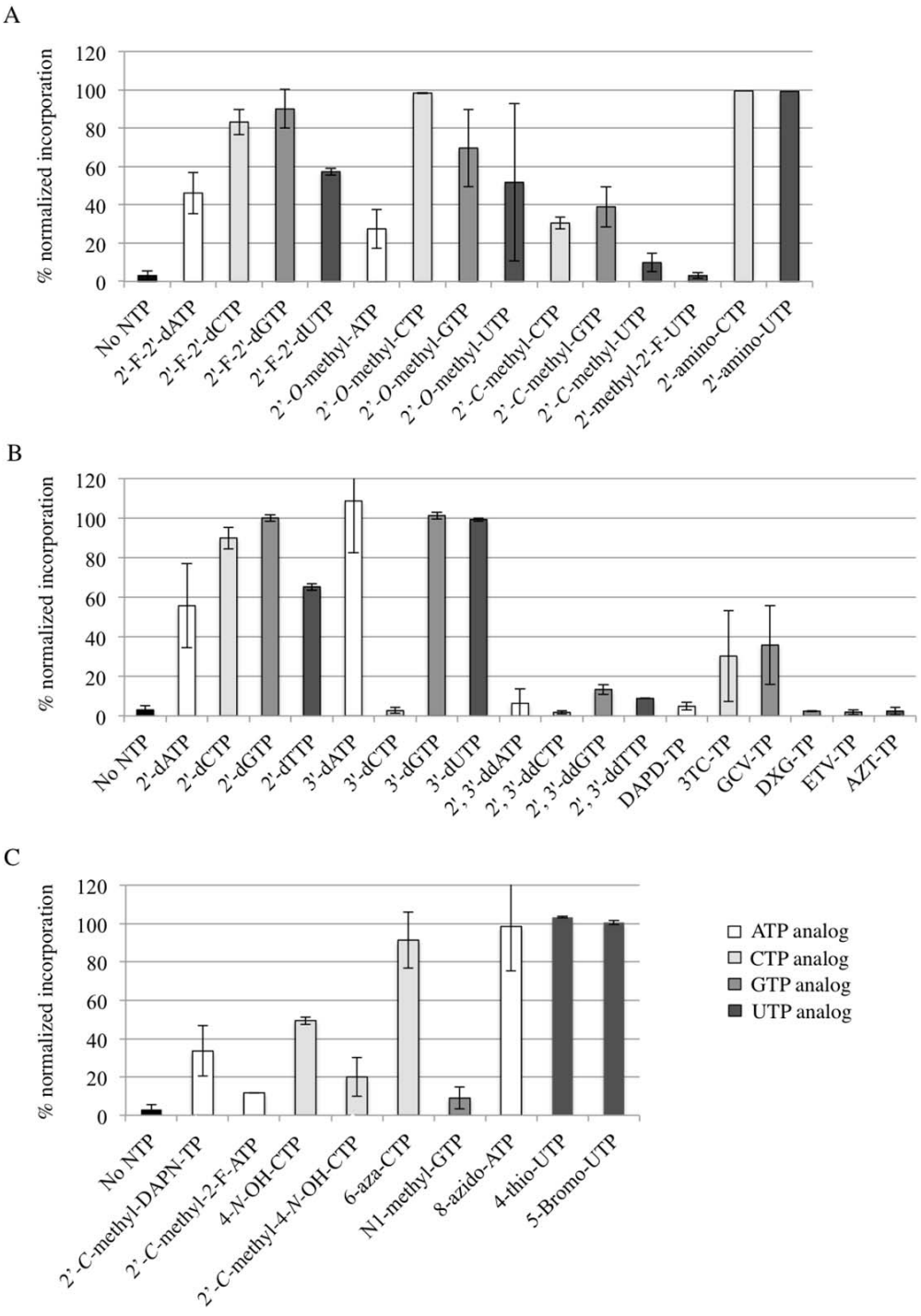


FIG 1. Chemical structures of NAI-TPs. A) Structures of NAI-TPs with modifications on the ribose moiety. Compounds are grouped according to base moiety. B) Chemical structures dNAI-TPs with anti-HIV, anti-HBV or anti-HSV activity. C) Structures of ribonucleoside analog 5'-triphosphates with modifications on the base moiety.



282

FIG 2. Nucleoside analog 5'-triphosphate incorporation by POLRMT. A) Nucleoside analog triphosphates with modifications at the 2'-position of the ribose moiety were assessed for incorporation by POLRMT. Incorporation reactions were allowed to proceed for 2 h in the presence of 100 μM of each substrate. Percentage incorporation for each nucleoside analog triphosphate was normalized to that of the corresponding natural nucleotide (ATP, CTP, GTP, or UTP) substrate. B) POLRMT incorporation of 2'-deoxyribonucleoside analogs and NAI-TPs with anti-HBV, anti-HIV or anti-HSV activity was assessed as described above. C) POLRMT incorporation was assessed for ribonucleoside analog 5'-triphosphates with modifications on the base moiety. Error bars represent S.D. values for two to three separate experiments.

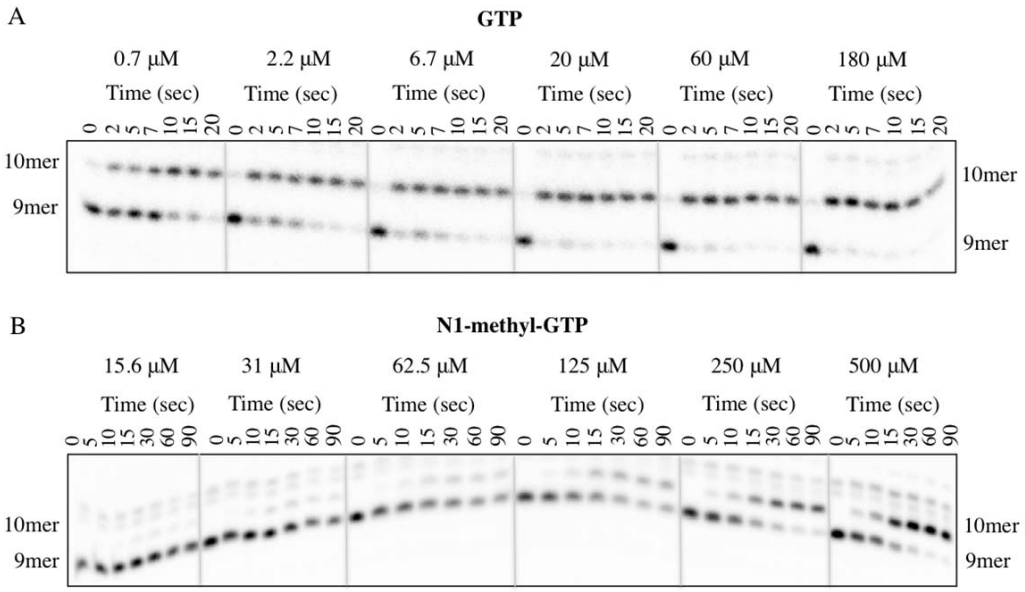


FIG 3. Incorporation profile of N1-methyl-GTP. A) Increasing concentrations of GTP (0.7 µM to 180 µM) were incubated with a pre-formed NS5B: RNA/RNA complexes and nucleotide extension was measured over time. The extended 10mer RNA product was visualized on a 20% denaturing acrylamide gel. Rates of incorporation at various nucleotide concentrations were plotted as described previously in order to obtain apparent dissociation constant ($K_{d,app}$) value of 4.1 ± 1.6 µM for GTP (17). B) Increasing concentrations of N1-methyl-GTP (15.6 µM to 500 µM) were incubated with the preformed NS5B:RNA/RNA complex as described above. $K_{d,app}$ value for N1-methyl-GTP was estimated to be > 100 µM.

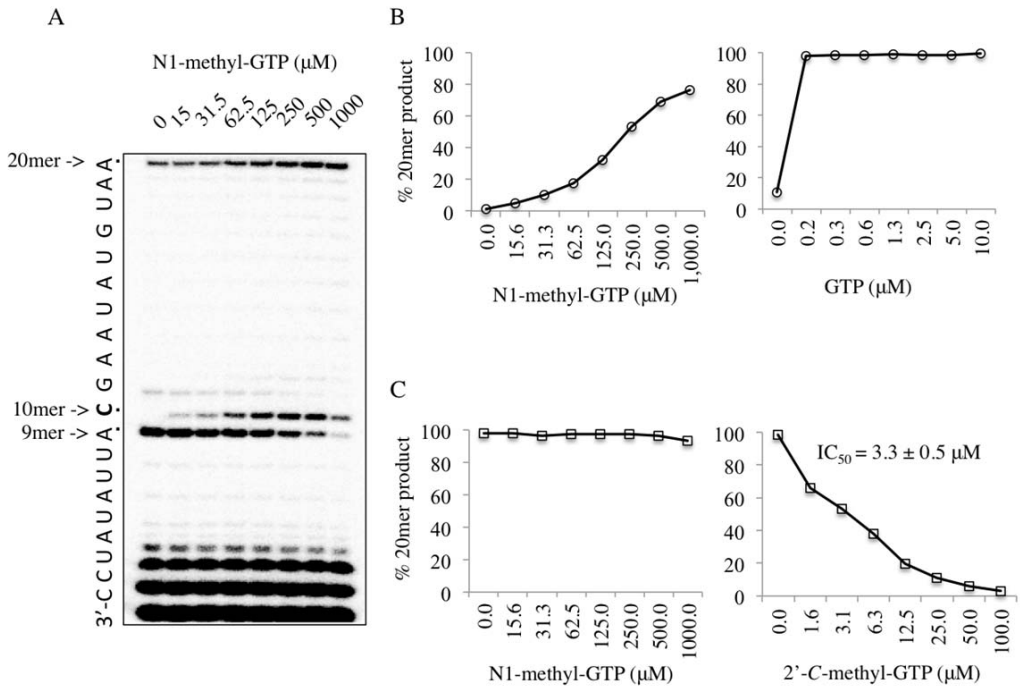
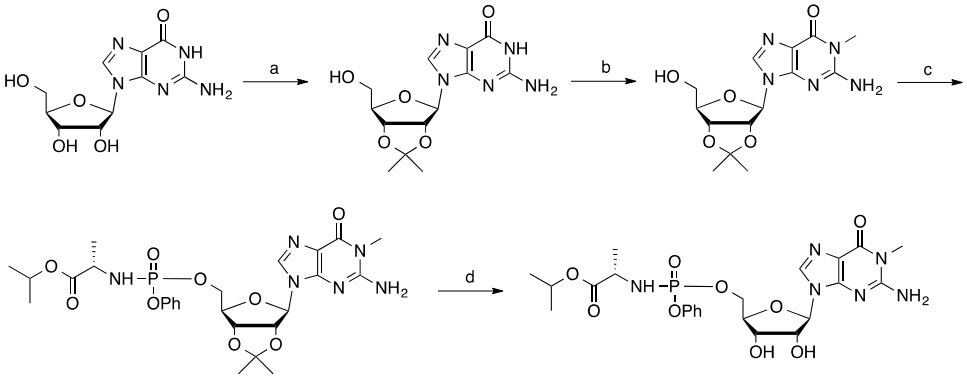


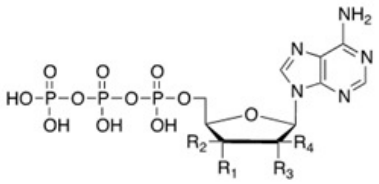
FIG 4. Effect of N1-methyl-GTP incorporation on NS5B-mediated RNA extension. A) Increasing concentrations of N1-methyl-GTP were incubated with NS5B enzyme, 5'-radiolabeled GG primer, and a 20mer RNA template in the presence of 10 μM ATP, CTP, and UTP. RNA synthesis was allowed to proceed for 2 h at 30°C. In the absence of N1-methyl-GTP (lane 0), a strong pausing site was observed at position 9 while small amounts of 20mer full-length RNA product were accumulated as a result of nucleotide misincorporation. Increasing N1-methyl-GTP concentrations correlated with the appearance of a 10mer band (site of G incorporation) and full-length 20mer product. B) Amount of 20mer product accumulation was plotted as a function of N1-methyl-GTP concentration (left). Parallel experiment with increasing concentrations of GTP was also plotted (right). C) RNA synthesis was monitored as described above, with the addition of 1 μM GTP in the presence of increasing concentrations of N1-methyl-GTP (left) and control inhibitor 2'-*C*-methyl-GTP (right). No inhibition of RNA

synthesis was observed with up to 1,000 µM of N1-methyl-GTP while 2'-*C*-methyl-GTP inhibited RNA synthesis with an IC_{50} value of 3.3 ± 0.5 µM (average of two separate experiments ±S.D.).

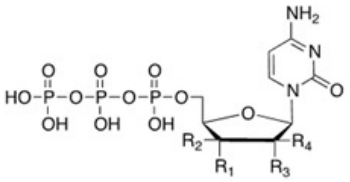


Scheme 1. Chemical synthesis of N1-methyl-G-phosphoramidate prodrug. (a) *p*-Toluenesulfonic acid, 2,2-dimethoxypropane, acetone, rt, overnight, 93%; (b) NaH, MeI, DMSO, rt, 89.7%; (c) phosphorochloridate, N-methylimidazole, THF/CH_3CN, rt, 3 h, 90.7%; (d) 85% TFA, rt, 1 h, 86%.

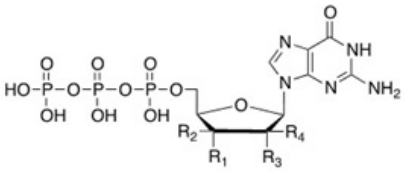
A



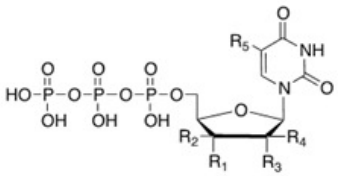
(1) ATP: R_1= OH, R_3= OH, R_2= R_4= H,
(2) 2'-dATP: R_1= OH, R_2= R_3= R_4= H
(3) 2', 3'-ddATP: R_1= R_2= R_3= R_4= H
(4) 3'-dATP: R_1= R_2= R_4= H, R_3= OH
(5) 2'-*O*-methyl-ATP: R_1= OH, R_2= R_4= H, R_3= OCH_3
(6) 2'-F-dATP: R_1= OH, R_2= R_4= H, R_3= F



(7) CTP: R_1= OH, R_2= R_4= H, R_3= OH
(8) 2'-dCTP: R_1= OH, R_2= R_3= R_4= H
(9) 2'-3'-ddCTP: R_1= R_2= R_3= R_4= H
(10) 2'-*O*-methyl-CTP: R_1= OH, R_2= R_4= H, R_3= OCH_3
(11) 2'-*C*-methyl-CTP: R_1= OH, R_2= H, R_3= OH, R_4= CH_3
(12) 2'-amino-CTP: R_1= OH, R_2= R_4= H, R_3= NH_2
(13) 3'dCTP: R1= R_2= R_4= H, R_3= OH
(14) 2'-F-dCTP: R_1= OH, R_2= R_4= H, R_3= F

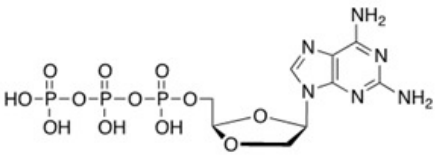


(15) GTP: R_1= OH, R_2= R_4= H, R_3= OH
(16) 2'-dGTP: R_1= OH, R_2= R_3= R_4= H
(17) 2', 3'-ddGTP: R_1= R_2= R_3= R_4= H
(18) 2'-*C*-methyl-GTP: R_1= OH, R_2= H, R_3= OH, R_4= CH_3
(19) 3'-dGTP: R_1= R_2= R_4= H, R_3= OH
(20) 2'-*O*-methyl-GTP: R_1= OH, R_2= R_4= H, R_3= OCH_3
(21) 2'-F-dGTP: R_1= OH, R_2= R_4= H, R_3= F

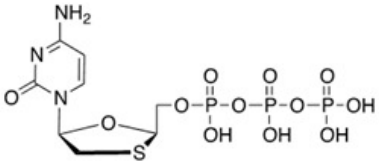


(22) UTP: R_1= OH, R_2= R_4= R_5= H, R_3= OH
(23) 2'-dTTP: R_1= OH, R_2= R_3= R_4= H, R_5= CH_3
(24) 2', 3'-ddTTP: R_1= R_2= R_3= R_4= H, R_5= CH_3
(25) 2'-C-methyl-UTP: R_1= OH, R_2= R_5= H, R_3= OH, R_4= CH_3
(26) 2'-amino-UTP: R_1= OH, R_2= R_4= R_5= H, R_3= NH_2
(27) 2'-*C*-methyl-2'-F-dUTP: R_1= OH, R_2= R_5= H, R_3= F, R_4= CH_3
(28) 3'-dUTP: R_1= R_2= R_4= R_5= H, R_3= OH
(29) 2'-*O*-methyl-UTP: R_1= OH, R_2= R_4= R_5= H, R_3= OCH_3
(30) 2'-F-dUTP: R_1= OH, R_2= R_4=R_5= H, R_3= F

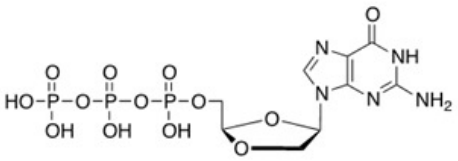
B



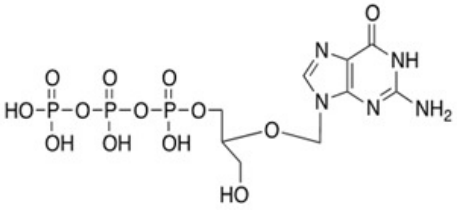
DAPD-TP
(2,6-diaminopurine dioxolane) (31)



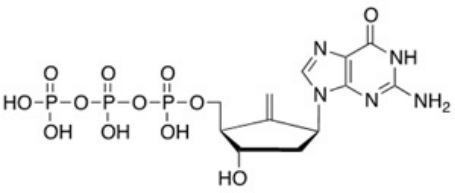
3TC-TP (lamivudine) (32)



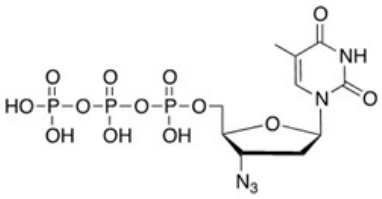
DXG-TP (guanosine dioxolane) (33)



GCV-TP (ganciclovir) (34)

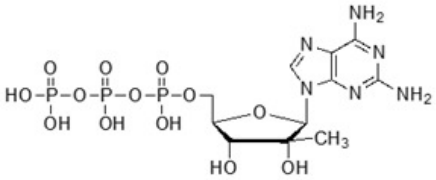


ETV-TP (entecavir) (35)

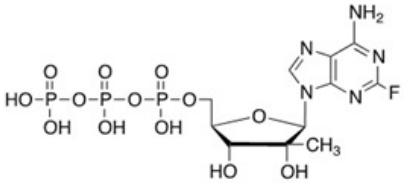


AZT-TP (zidovudine) (36)

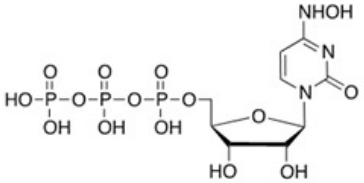
C



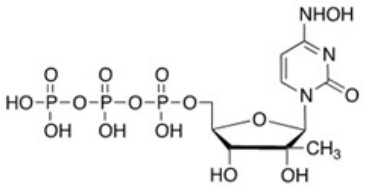
2'-*C*-methyl-DAPN-TP (37)



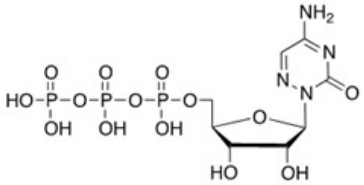
2'-*C*-methyl-2-F-ATP (38)



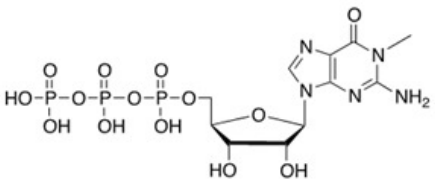
2'-*C*-methyl-C-4-*N*-OH-CTP (39)



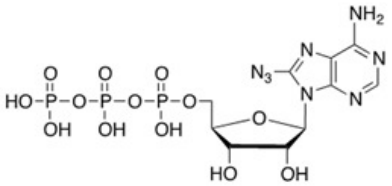
4-*N*-OH-CTP (40)



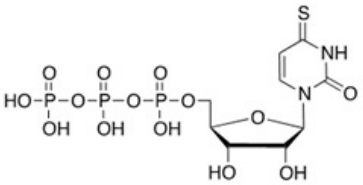
6-aza-CTP (41)



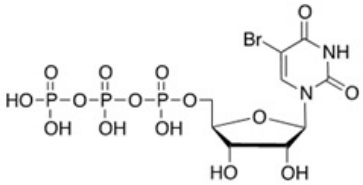
N1-methyl-GTP (42)



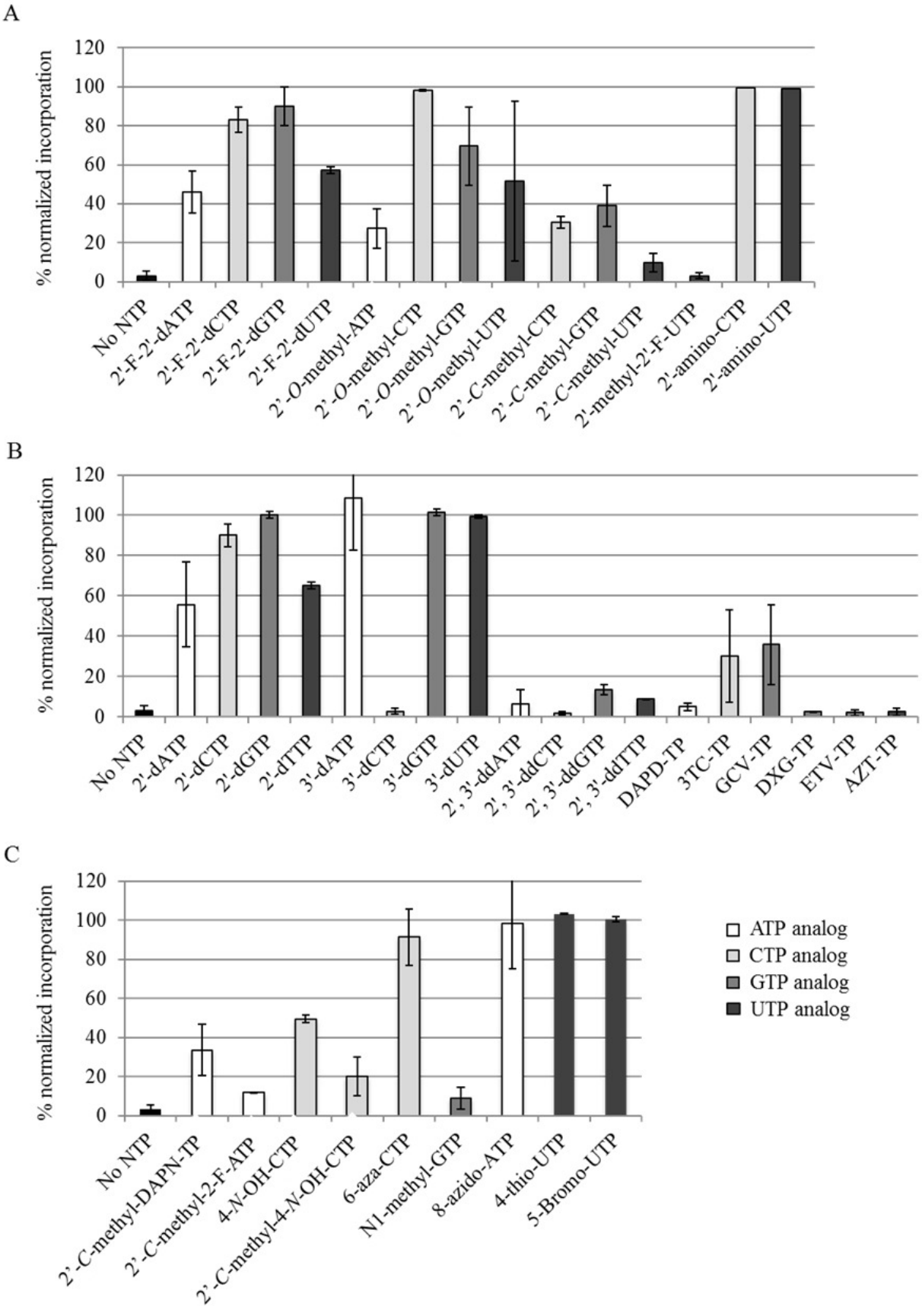
8-azido-ATP (43)

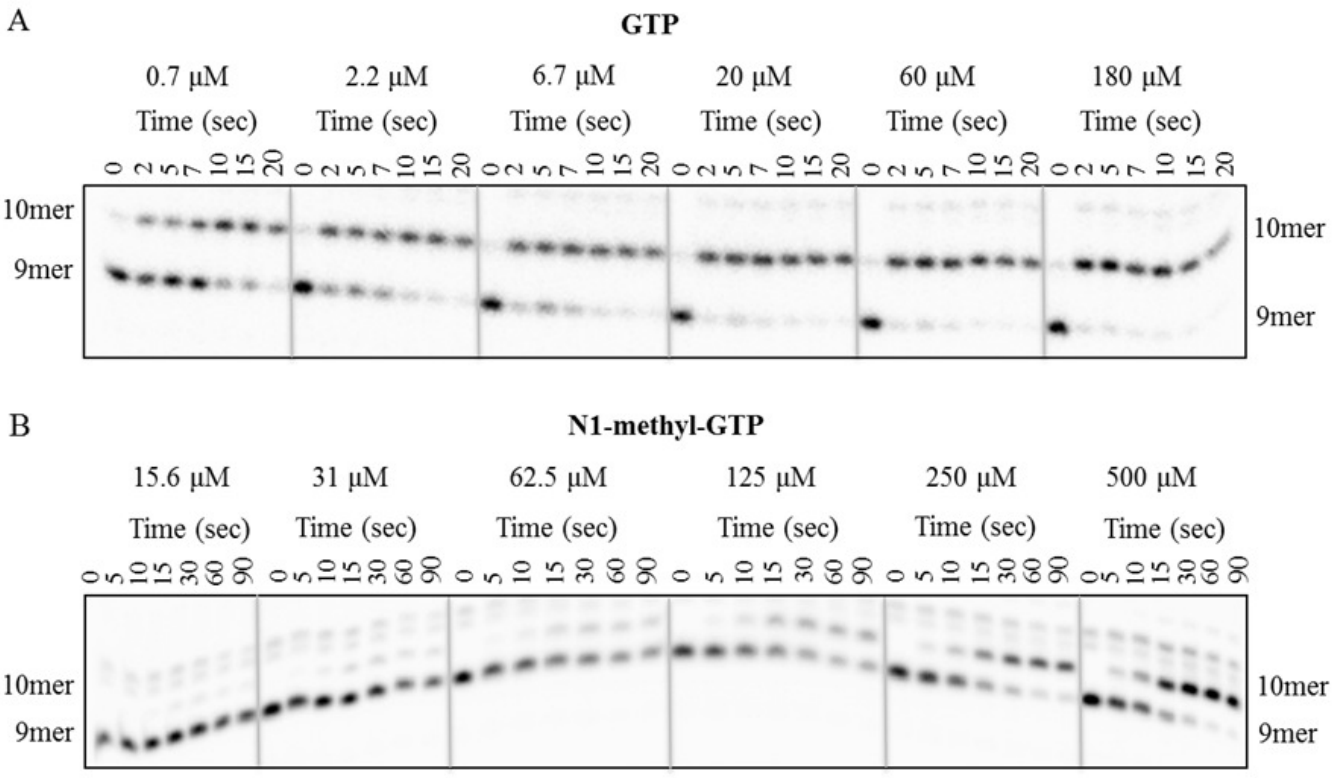


4-thio-UTP (44)



5-bromo-UTP (45)





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