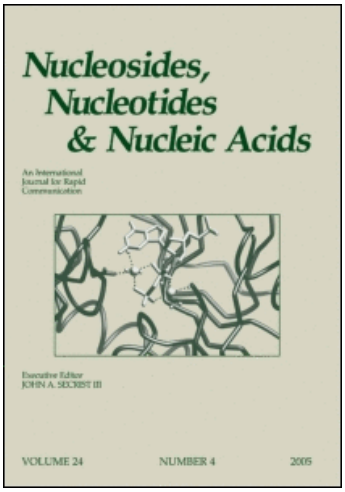

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Mode of Inhibition of HIV Reverse Transcriptase- Catalyzed DNA Synthesis by 3'-Amino-3'-Deoxythymidine 5' -Tfiphosphate

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MODE OF INHIBITION OF HIV REVERSE TRANSCRIPTASE-CATALYZED DNA SYNTHESIS BY 3'-AMINO-3'-DEOXYTHYMIDINE 5'-TRIPHOSPHATE

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Abstract. The molecular mode of action of 3'-amino-3'-deoxythymidine 5'-triphosphate in the polymerization reaction catalyzed by human immunodeficiency virus reverse transcriptase was elucidated. This compound was shown to be a termination substrate on both DNA and RNA templates.

Abbreviations are: AMV and HIV - avian myeloblastosis and human immunodeficiency viruses; ddNTP(3'NH_2) - 3'-amino-2',3'-dideoxynucleoside 5'-triphosphate; ddNTP(3'N_3) - 3'-azido-2',3'-dideoxynucleoside 5'-triphosphate, ddNTP - 2',3'-dideoxynucleoside 5'-triphosphates; d_4TTP - - 2',3'-didehydro-2',3'-dideoxynucleoside 5'-triphosphate, ddNTP(3'F) - 3'-fluoro-3'-deoxythymidine 5'-triphosphate.

Introduction

Over the last years, several papers have appeared dealing with the molecular mechanism of action of ddNTP(3'NH_2) containing natural nucleic bases, including ddTTP(3'NH_2). It has been shown that ddNTP(3'NH_2) are

This paper is dedicated to the 75th birthday of Dr.Yoshihisa Mizuno

terminating substrates of DNA synthesis catalyzed by mammalian DNA polymerases α and β, as well as *E.coli* DNA polymerase I[1]. Similar results were observed for AMV reverse transcriptase[2] and some other DNA polymerases[3-5] and recombinant HIV reverse transcriptase[6]. In all these works, different natural DNA templates were used. In contrast to these results, it has recently been shown[7] that ddTTP(3'NH_2) inhibits [poly(rA)-oligo(dT)]-dependent poly(dT) synthesis catalyzed by the HIV reverse transcriptase noncompetitively against dTTP. The reaction kinetics was linear and differed from that for ddTTP(3'N_3). Moreover, ddTTP(3'NH_2) was not incorporated into the 3' termini of oligo(dT) and did not terminate oligo(dT) elongation. We assume that ddTTP(3'NH_2) did not terminate oligo(dT) elongation because [poly(rA)-oligo(dT)] was used as the template-primer.

To resolve this contradiction, we studied the substrate properties of ddTTP(3'NH_2) using two template-primers, namely M13mp10 DNA-oligodeoxynucleotide (DNA-dependent system) and pPV-19 transcript - oligodeoxynucleotide (RNA-dependent system). Both the single-substrate and complete systems [four natural dNTPs + ddTTP(3'NH_2)] were used to elucidate the molecular mode of ddTTP(3'NH_2) action. It was shown that the nucleotide residue of ddTTP(3'NH_2) is incorporated into the primer 3' terminus in both systems and prevents subsequent DNA elongation.

Experimental

Materials and Methods. Phage M13mp10 DNA was isolated from the cultural fluid of *E.coli* K12XL1[8]. Heterodimeric RNA was synthesized by run-off transcription of *Sal*G1-digested plasmid pPV-19 with T7 RNA polymerase[9]. Plasmid pPV-19 containing a 120-bp fragment of pBR322 DNA between the *Sal*G1 and *Sph*I sites was a kind gift of Dr. S.Kochetkov. Oligodeoxynucleotide primers (Scheme 1) were labeled at the 5' position by

a.

```
                    10         20         30         40
                    .          .          .          .
      3'   GGGTCAGTGCTGCAACATTTTGCTGCCGGTCACGGTTCGAACCCGACGT
[5'-32P]   CCCAGTCACGACGT
           50         60         70         80         90
           .          .          .          .          .
           CCAGCTGAGATCTCCTAGGGGCCCGCTCGAGCTTAAGCATTAGTACCA
```

b.

```
                        10         20         30         40
                        .          .          .          .
          3'  CGAGAGGGAAUACGCUGAGGACGUAAUCCUUCGUCGGGUCAUCAU
1   [5'-32P]  GCTCTCCCTTATGCGACTCC
2   [5'-32P]            ATGCGACTCCTGCA

                50         60         70         80
                .          .          .          .
           CCAACUCCGGAAACUCGUGGCGGCGGCGUUCCUUACCACGUAG
```

Scheme 1

T4 polynucleotide kinase (Amersham) using [γ-^{32}P] ATP (specific activity 1500 Ci/mmol, Radioizotop, Russia). After phenol extraction, the labeled oligonucleotides were twice precipitated with ethanol, dissolved in water to 1 μM, and annealed with templates. The template-primer complexes were purified on BioGel A1.5m. AMV reverse transcriptase was obtained from Omutninsk Chemicals (Russia), HIV reverse transcriptase was isolated as shown earlier[6].

Modified nucleoside 5'-triphosphate were synthesized as previously described: ddNTP(3'NH_2) and ddNTP(3'N_3)[10], d_4TTP[11], and ddTP(3'F)[12]. ddNTPs were from Sigma.

Reverse transcriptase assays. Extension of the oligonucleotide annealed to M13mp10 DNA (Scheme 1a) was carried out as described earlier[13]. Extension of oligonucleotide annealed to the RNA template was performed using the RNA sequencing technique as described previously[14,15].

Determination of kinetic constants. The assay mixture (6 μl) contained 0.1 μM ^{32}P-labeled RNA primer-complex (Scheme 1,b1), 1-2 units of AMV or HIV reverse transcriptase, 50 mM Tris-HCl (pH 7.2, 8.2, or 9.2), 50 mM KCl, 10 mM $MgCl_2$, 10 mM DTT, 5% glycerol, 0.25% Triton X100, one of the dNTP analogs at different concentrations (0.005-15 μM), and 0.2 unit/μl RNasin ribonuclease inhibitor (Omutninsk Chemicals, Russia). The reaction was carried out for 2.5 min at 37°C and stopped by adding 3 μl of deionized formamide containing 20 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol. The products were boiled for 5 min and separated by electrophoresis in denaturing 20% polyacrylamide gel containing 8M urea. The products were quantitated by exposing XRP-5 Kodak films, followed by scanning the product bands in an LKB scanner. The concentration dependences of the incorporation rate for dNTP analogs were used to construct the Lineweaver-Burk plot and determine the kinetic constants[16].

The specific activity of HIV reverse transcriptase is 54,000 un./mg; one activity unit is determined as the amount of enzyme catalyzing incorporation of 1 nmole of dTMP in poly(rA)-oligo(dT) in 10 min as 37°C.

Results

Termination of oligodeoxynucleotide elongation by ddTTP(3'NH_2) on DNA template. The results of experiments with the DNA template (Scheme 1a) are shown in Fig.1. The experiment was carried out at pH 8.2; ddTTP, ddTTP(3'F), and ddTTP(3'N_3) were used in control assays. All compounds at definite concentrations (different for each compound) produced a chain termination (T32, T40, T41, T46, T52, T57, T59, T66, T79, and other) according to the template used.

Figure 2 demonstrates a similar experiment, which was also performed using the DNA template and HIV reverse transcriptase at pH 7.2, 8.2, and

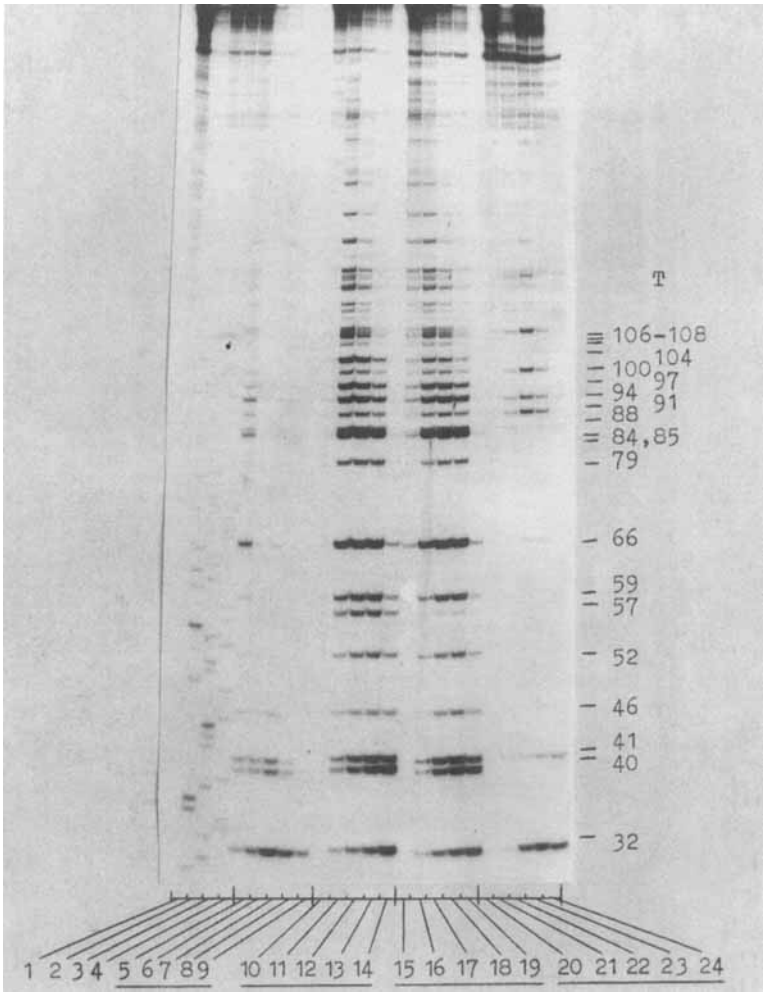

Figure 1. PAGE separation of the primer elongation products at pH 8.2 on the DNA template (Scheme **1a**) catalyzed by HIV reverse transcriptase in the presence of ddNTP, ddNTP(3'NH_2), ddTTP(3'F), or ddTTP(3'N_3). Track **1** - 0.02 μM DNA-primer complex + 20 μM dNTPs (control); **2-4** - as in **1** + 1 μM dATP + 20 μM dGTP, dCTP, and dTTP + 0.8 μM ddATP (**2**), 1 μM dGTP + 20 μM dATP, dCTP, and dTTP + 0.8 μM ddGTP (**3**), 1 μM dCTP + 20 μM dATP, dGTP, and dTTP + 0.8 μM ddCTP (**4**); **5-24** - as in **1** + 1 μM dTTP + 20 μM dATP, dGTP, and dCTP + ddTTP (**5-9**), ddTTP(3'NH_2) (**10-14**), ddTTP(3'F) (**15-19**), or ddTTP(3'N_3) (**20-24**). The concentration of the terminating substrates was 0.2 μM (**5**, **10**, **15**, **20**), 2 μM (**6**, **11**, **16**, **21**), 5 μM (**7**, **12**, **17**, **22**), 10 μM (**8**, **13**, **18**, **23**) and 50 μM (**9**, **14**, **19**, **24**).

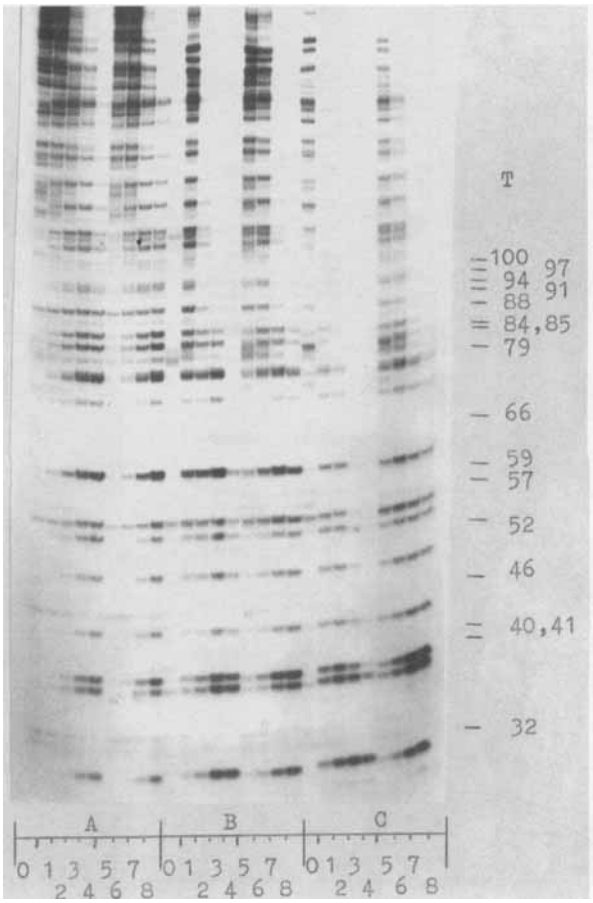

Figure 2. PAGE separation of the primer elongation products on the DNA template (Scheme **1a**) catalyzed by HIV reverse transcriptase at pH 7.2 (series **A**), 8.2 (**B**) and 9.2 (**C**) in the presence of ddTTP(3'NH_2) (**1-4**) and ddTTP(3'N_3) (**5-8**). The assay mixture contained 0.02 μM DNA-primer complex, 1 μM dTTP, 20 μM dATP, dGTP, and dCTP. The concentration of the terminating substrates was 2 μM (**1, 5**), 5 μM (**2, 6**), 10 μM (**3, 7**), and 50 μM (**4, 8**). 0 - control without terminating substrates.

9.2. Two compounds, ddTTP(3'NH_2) and ddTTP(3'N_3), are compared. The termination picture was similar for both compounds. The termination bands were the same as in Fig.1. Upon raising the pH to 9.2, the termination efficiency increased.

Termination of oligodeoxynucleotide elongation by ddTTP(3'NH_2) on RNA template. It can be seen in Fig.3 that the nucleotide residue of ddTTP(3'NH_2) is incorporated into the oligonucleotide on the RNA template (Scheme 1, b2) at positions 15, 16, 31, 34, 38, 39, 48 and 49 (tracks 8,9) starting from the primer 5' terminus, i.e., against adenine residues of the template. In the control assays, ddTTP (tracks 6,7) and ddTTP(3'N_3) (tracks 10,11) were used. At all pH values, the termination efficiency depended on the ddTTP(3'NH_2) concentration. The electrophoretic mobility of the ddTTP(3'NH_2)-terminated oligonucleotides was different from that for ddTTP- and ddTTP(3'N_3)-terminated ones (bands T15 and T16). This effect was caused by the positive charge of the NH_3^+ group. The presence of T15 and T16 bands in control tracks 2-5 was due to stops at these positions.

Very similar results were obtained for ddTTP(3'NH_2), ddTTP, and ddTTP(3'N_3) in the reaction catalysed by AMV reverse transcriptase in the same system (data not shown).

Figure 4 demonstrates primer (Scheme 1, b2) elongation by the same terminating substrates catalyzed by both HIV (4a) and AMV (4b) reverse transcriptases at pH 8.2 in the absence of natural dNTPs. Clearly, the elongation efficiency depended on the concentration of the terminating substrates. Electophoretic mobility of the resulting pentadecadeoxynucleotides was different. It is evident from the termination pictures that HIV reverse transcriptase incorporated ddTTP(3'NH_2) and ddTTP(3'N_3) more efficiently as compared with the AMV enzyme. This observation is supported by the kinetic data: for HIV reverse transcriptase, the K_m values were 3-10 times lower (Table 1).

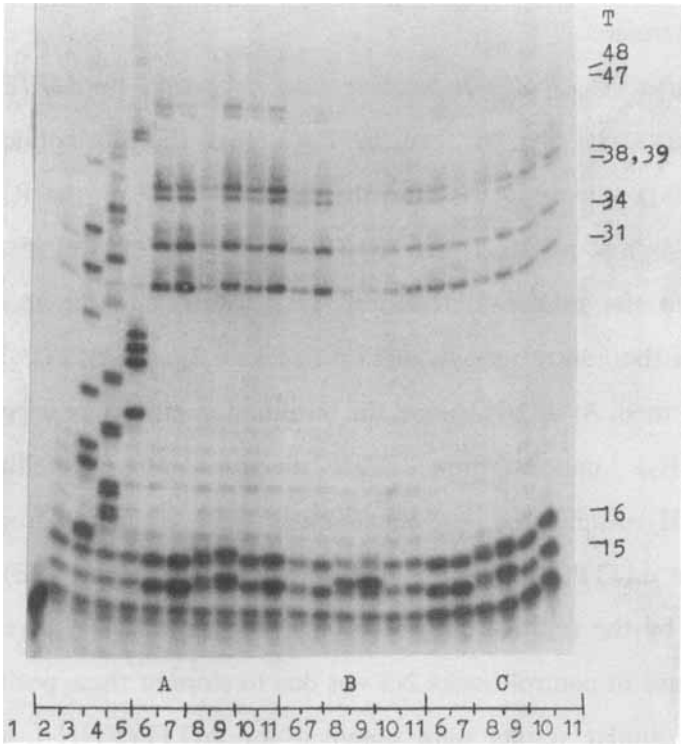

Figure 3. PAGE separation of the primer elongation products on the RNA template (Scheme **1**, **b2**) catalyzed by HIV reverse transcriptase (3 activity units, 1 h, 37°C) at pH 8.2 (**A**), 7.2 (**B**), and 9.2 (**C**). Track 1 - 0.16 μM RNA-primer complex **b2**; 2 - primer elongation in the presence of 125 μM dNTPs; 3-5 - as in 1 + three 125 μM dNTPs and 25 μM fourth dNTP + 20 μM base-analogous ddNTP: dATP + ddATP (**3**), dGTP + ddGTP (**4**), dCTP + ddCTP (**5**); **6-11** - as in 1 + 125 μM dATP, dGTP, and dCTP + 25 μM dTTP + 20 and 100 μM ddTTP (**6**, **7**), or ddTTP(3'NH_2) (**8**, **9**), or ddTTP(3'N_3) (**10**, **11**). The length of the resulting fragments is shown in the right.

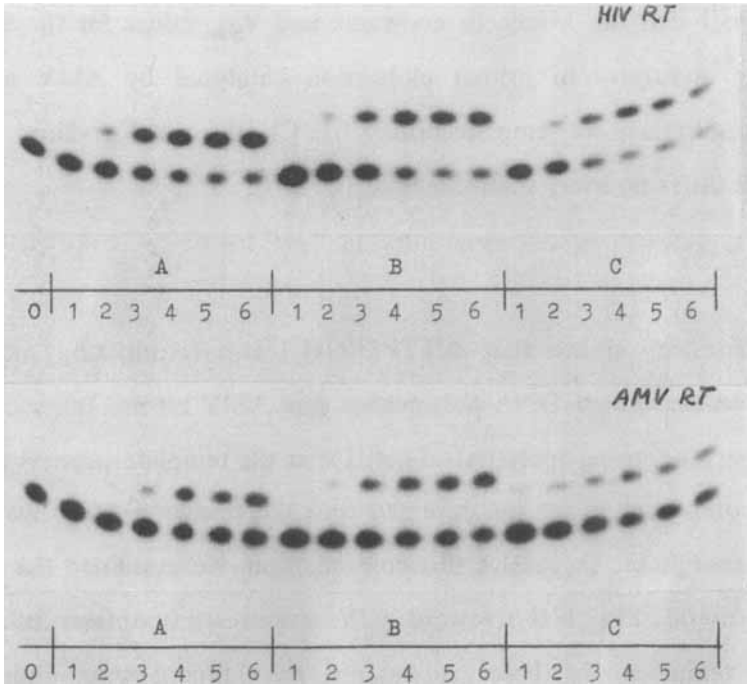

Fig. 4. PAGE separation of the primer elongation products on the RNA template (Scheme 1, b2) catalyzed by HIV and AMV reverse transcriptases (1.2 and 1.7 activity units, respectively; 2.5 min, 37°C, pH 8.2). Track 0 - 0.16 μM template-primer complex b2; 1-6 - as 0 + 0.01, 0.04, 0.2, 1, 4, and 12 μM ddTTP (series A), ddTTP(3'NH_2) (B), and ddTTP(3'N_3) (C).

Table 1. The K_m values and V_{max}/V_{max}(dTTP) ratios for ddTTP, d_4TTP, ddTTP(3'NH_2) and ddTTP(3'N_3) in primer elongation on the RNA template (Scheme 1, b1) catalyzed by HIV and AMV reverse transcriptases at pH 8.2

	HIV reverse transcriptase		AMV reverse transcriptase	
Compound	K_m, μM	V_{max}/V_{max}(dTTP)	K_m, μM	V_{max}/V_{max}(dTTP)
dTTP	0.0075± 0.0005	1.00	0.0282± 0.0018	1.00
ddTTP(3'NH_2)	0.0319± 0.0049	0.77	0.0945± 0.0048	0.51
ddTTP(3'N_3)	0.0247± 0.0006	0.92	0.0647± 0.0068	0.90
ddTTP	0.0116± 0.0012	0.91	0.1179± 0.0455	0.55
d_4TTP	0.0204± 0.0022	0.84	0.1826± 0.0254	0.69

Table 1 lists the Michaelis constants and V_{max} values for the examined terminating substrates in primer elongation catalyzed by AMV and HIV reverse transcriptases on template-primer b1. Clearly, the K_m values for these compounds differ no more than threefold.

Discussion

It has been shown that ddTTP(3'NH_2) is a terminating substrate of several DNA-dependent DNA polymerases and AMV reverse transcriptases[2-5]. At the same time, using [poly(rA)-oligo(dT)] as the template-primer[7] found that this compound is not incorporated into the primer 3' terminus by HIV reverse transcriptase. To resolve this contradiction, we examined the substrate properties of ddTTP(3'NH_2) toward HIV reverse transcriptases using DNA and RNA templates. In these experiments, recombinant heterodimeric HIV reverse transcriptase (ratio of p66 and p51 1:1[6] was used. This enzyme has been shown to incorporate several 3'-modified dNTPs, including ddTTP(3'NH_2) into the primer on M13mp10 DNA as the template[6].

We reproduced the data[6] and showed that ddTTP(3'NH_2) terminates DNA-dependent DNA synthesis catalyzed by HIV and AMV reverse transcriptases (Figs.1 and 2). Similar results were obtained for the RNA template (Figs. 3 and 4) It is evident from Table 1 that the Michaelis constants for ddTTP(3'NH_2), ddTTP(3'N_3), ddTTP, and d_4TTP are close to each other .

It seemed interesting to study primer elongation by ddTTP(3'NH_2) at different pH, because ddTTP(3'NH_2) exists in the reaction media in two equilibrium forms:

$$\text{ddTTP(3'NH}_2\text{)} \rightleftharpoons \text{ddTTP(3'NH}_3^+\text{)}$$

The ratio of these forms is determined by pH of the reaction mixture. The pK_a

value of the amino group in 3'-amino-3'-deoxythymidine 5'-phosphate was found to be 9.1-9.2[17]. Consequently, at pH 9.2 about 50% of ddTTP(3'NH_2) exists in the ddTTP(3'NH_3^+) form, whereas at pH 8.2 and at pH 7.2 the portion of ddTTP(3'NH_3^+) is about 5% and 0.5%, respectively. At all three pH values, HIV reverse transcriptase can catalyze DNA synthesis (Figs.2 and 3), though its activity is maximal at pH 8.2. It remains unclear which form of ddTTP(3'NH_2) terminates DNA synthesis. Presumably, both of them are active. Thus, in our opinion, the complex kinetics observed previously[7] is due to the existence of two forms. The contradiction between our results and the data of Kedar at al.[7] is presumably due to a low affinity of terminating substrates, including ddTTP(3'N_3) and ddTTP(3'NH_2) to [poly(rA)-oligo(dT)]. Indeed, the K_m value for ddTTP(3'N_3) in oligo-(dT) extention was 2.9 μM[7], whereas in our system K_m for ddTTP(3'N_3) was 0.0247 μM (Table 1).

The Michaelis constant values for ddTTP(3'NH_2) were only slightly higher than those for ddTTP, d_4TTP, and ddTTP(3'N_3), suggesting that all these compounds inhibit DNA synthesis by the same mechanism though the kinetic patterns of their interaction with the [template-primer + enzyme] complex are different.

Studying the molecular mechanism of ddTTP(3'NH_2) action seems to be important also for the reason that 3'-azido-3'-deoxythymidine (AZT) used for treatment of AIDS patients is partly reduced to 3'-amino-3'-deoxythymidine[18-20]. In human cell the latter compound is capable of being converted to ddTTP(3'NH_2)[21-23], which can be one of the main reasons for toxicity of AZT[18].

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