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Modified nucleoside 5′-triphosphates containing 2′,3′-fused three-membered rings as substrates for different DNA polymerases

Dimitry G. Semizarov, Lyubov S. Victorova, Alexander A. Krayevsky and Marina K. Kukhanova

Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 32 Vavilov St, Moscow 117984, Russian Federation

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5′-Triphosphates of 1-(2′,3′-epithio-2′,3′-dideoxy-β-D-lyxofuranosyl)thymine, 1-(2′,3′-epithio-2′,3′-dideoxy-β-D-ribofuranosyl)thymine and 2′,3′-lyxoanhydrothymidine have been shown to be termination substrates for human immunodeficiency virus (HIV) and avian myeloblastosis virus (AMV) reverse transcriptases as well as DNA polymerase I from *E. coli* and DNA polymerase β from rat liver. At the same time they do not terminate DNA synthesis catalysed by DNA polymerase ε from human placenta. K_m values of ltTTP, rtTTP and laTTP incorporation into the DNA chain during catalysis by AMV reverse transcriptase agree closely with each other being 1.5–2.5 times higher than K_m value for dTTP. Furthermore, V_{max} values for modified substrates are only 2–3 times lower than V_{max} for dTTP. The evidence favours the hypothesis of high affinity of modified nucleotides with a flattened furanosyl ring for DNA polymerase active sites.

DNA polymerase; Termination substrate; Modified nucleoside 5′-triphosphate

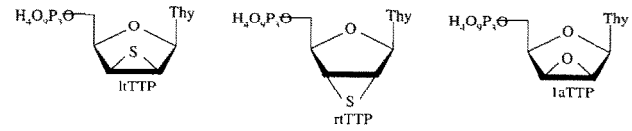
1. INTRODUCTION

In 1987 we showed d_4TTP to be termination substrate for a series of DNA polymerases [1,2]. Subsequently d_4CTP [3] and carbovirTP [4,5] were found to possess these properties. Restricted glycon conformational flexibility and high coplanarity of C1′, C2′,C3′ and C4′ atoms are distinguishing features of these compounds. That was demonstrated by X-ray analysis of unsaturated at C2′-C3′ bond in d_4NTP with adenine [6], cytosine [7], thymine [8,9] and guanine [10] as nucleic bases. The flattened glycone structure in d_4TTP and d_4CTP imitates the dNTP conformation in the [DNA polymerase + template-primer] complex [11].

Creation of an extra 2′,3′-fused three-membered ring also endows the glycon structure with a certain planarity and restricts its conformational flexibility as determined by X-ray analysis of 2′,3′-lyxoanhydrothymidine [12] and 2′,3′-riboanhydroadenosine [13]. NMR analysis in solution and conformational calculations using molecular mechanics methods of these compounds as well as 1-(2′,3′-epithio-2′,3′-dideoxy-β-D-lyxofuranosyl)uracil have shown that despite the flattened glycone structure their conformations are slightly different [14]. Furthermore, the presence of oxygen atoms of the epoxy ring and the bulky sulfur atoms of the epithiocycle in the *endo*-orientation (for lyxo-series) or in the *exo*-orientation (for ribo-series) as well as a C2′-C3′-bond length change as compared to dTTP can also influence the process of such nucleoside 5′-triphosphates binding to DNA polymerase active sites and thus alter their substrate properties.

Our earlier results [15,16] indicate that substrate properties of laTTP and laCTP are, first, different from properties of d_4TTP and, second, distinct from each other.

Presented here are some data on substrate properties of ltTTP, rtTTP and laTTP with respect to DNA polymerases from a variety of origins.



2. MATERIALS AND METHODS

DNA polymerases α and ε were isolated from human placenta according to [17], DNA polymerase β from rat liver was isolated as described in [18], *E. coli* DNA polymerase I Klenow fragment was from Amersham (UK), AMV reverse transcriptase from Omutninsk Chemicals (Russia). HIV reverse transcriptase was a kind gift of Dr T. Rosovskaya. DNA polymerase assays were performed as described in [19]. ltTTP and rtTTP were kind gifts of Dr M. Jasko, laTTP was synthesized according to [20]. For the kinetic measurements the AMV reverse transcriptase assay (6 μl) contained 0.01 mM ^{32}P-labeled template–primer complex, the enzyme (3 activity units), 10 mM Tris-HCl (pH 8.2), 5 mM $MgCl_2$, 40 mM KCl, 1 mM DTT, and the modified substrate or dTTP. The assay mixture was incubated for 3 min at 37°C and terminated by the addition of formamide with EDTA and dyes. Reaction products were separated by PAGE and gel radioautographs were then analyzed by scanning densitometry.

Correspondence address: D.G. Semizarov, Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, 32 Vavilov St, Moscow 117984, Russian Federation.

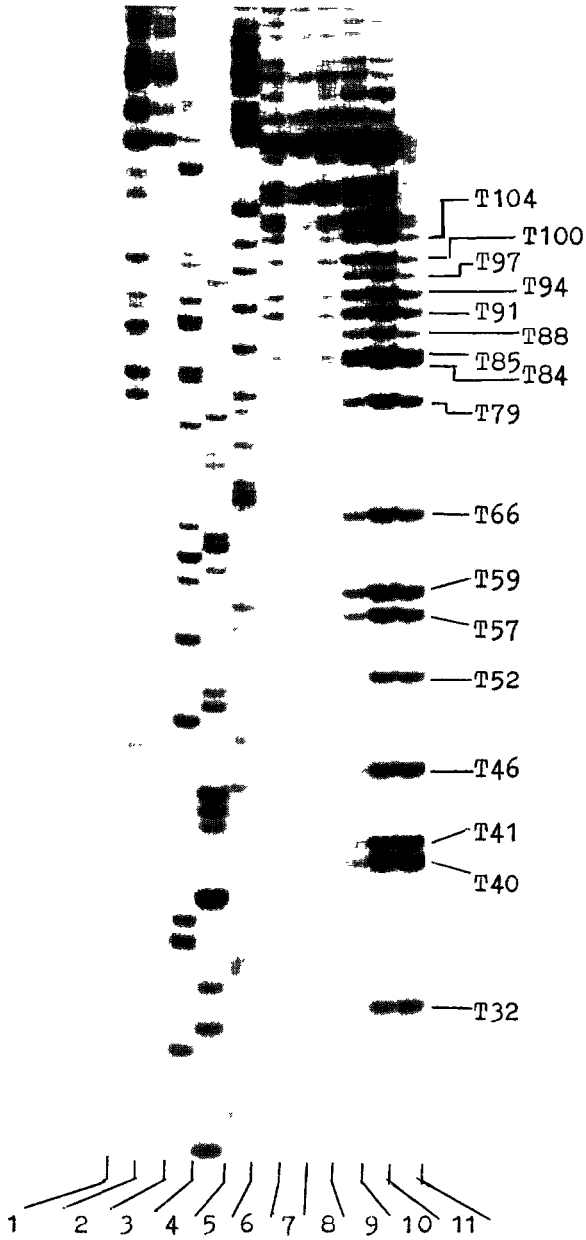


Fig. 1. PAGE separation of the products of primer elongation catalyzed by AMV reverse transcriptase. Tracks 1,2: synthesis in the absence of termination substrates (control). Tracks 3–6: synthesis in the presence of ddATP, ddGTP, ddCTP and ddTTP, respectively Tracks 7–11: in the presence of ltTTP at the following concentrations: 0 5 μM (7), 2 μM (8), 5 μM (9), 20 μM (10), 50 μM (11). Assays 2–10 were subsequently chased with an excess of four dNTPs.

3. RESULTS

For investigation of the ltTTP, rtTTP and laTTP substrate properties we employed several variations of cell-free systems containing DNA polymerases α and ε from human placenta, β from rat liver, I KF from *E. coli* as well as HIV and AMV reverse transcriptases. M13mp10 phage single-stranded DNA was selected as the template, 14-membered oligodeoxyribonucleotide served as the primer (as in [19]).

Table I

Termination of DNA synthesis catalyzed by several DNA polymerases

Analog	Reverse transcriptases		DNA polymerases			
	AMV	HIV	α	β	ε	I
ltTTP	+	+	+	+	–	+
rtTTP	+	+		+	–	+
laTTP	+	+		+	–	+

(+), incorporation into the primer 3′-terminus, (–), no incorporation

ltTTP, rtTTP, and laTTP were found to terminate DNA synthesis catalysed by AMV reverse transcriptase (Fig. 1, tracks 7–11, data on rtTTP and laTTP not shown). All bands of these tracks accord with the reference tracks 6 which represent termination by ddNTP. It can be seen that termination becomes more pronounced with increasing concentrations of ltTTP, rtTTP, and laTTP. A similar situation takes place when synthesis catalysed by HIV reverse transcriptase is terminated by ltTTP (Fig. 2, tracks 7–12) as well as rtTTP and laTTP (data not shown). All of the tested modified nucleoside 5′-triphosphates have been shown to terminate DNA synthesis catalysed by DNA polymerase I from *E. coli* (Fig. 3 represents termination by ltTTP). Results obtained for all of the examined DNA polymerases are given in Table I.

Inhibition of DNA synthesis by compounds under study in the system containing four natural substrates dNTP was also measured. The obtained inhibitor concentration:base-analogous substrate concentration ratios whereby DNA synthesis is inhibited by 50% are summarised in Table II. From this table it will be obvious that all of the tested compounds inhibit DNA synthesis catalysed by different DNA polymerases, the most marked inhibition being observed for HIV reverse transcriptase. At the same time inhibition of human

Table II

Inhibitor: substrate concentration ratios whereby DNA synthesis is inhibited by 50%

Compound	Reverse transctiptases		DNA polymerase α
	AMV	HIV	
ltTTP		3	100
rtTTP	20	1.4	20
laTTP	20	1.7	20

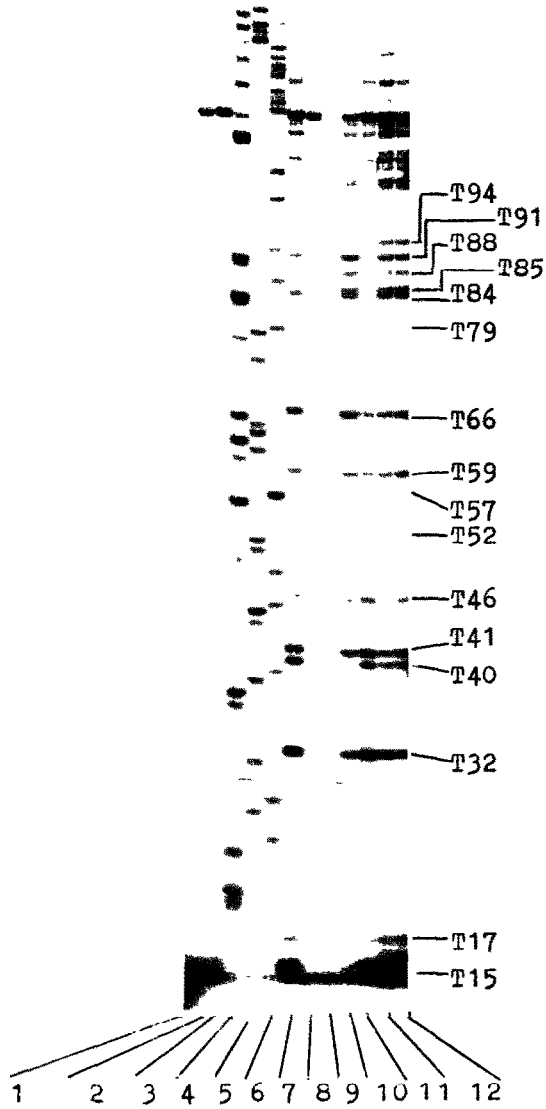


Fig. 2. PAGE separation of the products of primer elongation catalyzed by HIV reverse transcriptase. Tracks 1,2: synthesis in the absence of termination substrates (control). Tracks 3–6: synthesis in the presence of ddATP (3), ddGTP (4), ddCTP (5) and ddTTP (6), respectively. Tracks 7–12: in the presence of ltTTP at the following concentrations: 5 μM (7), 20 μM (8), 50 μM (9), 100 μM (10), 300 μM (11), 600 μM (12). Assays 2–12 were subsequently chased with an excess of four dNTPs.

DNA polymerase α by these compounds is weaker by one order of magnitude. We also measured the kinetic constants of the primer elongation reaction catalysed by AMV reverse transcriptase. We used a single substrate system, ltTTP, rtTTP and laTTP as well as dTTP were employed as substrates. At the outset we studied the time dependence of 15-mer formation and identified the linear portion of the rate–time curve. All the subsequent experiments were performed within this time lapse, optimal reaction duration was 3 min. We next studied the dependence between the rate of product formation and substrate concentration for ltTTP, rtTTP, laTTP as well as dTTP. K_m values and V_{max}/V_{max}(dTTP) ratios for all three substrates are given in Table III. It can be seen from Table III that K_m values for ltTTP, rtTTP, and laTTP incorporation into the DNA chain are in close agreement with each other and are not too different from K_m for dTTP.

4. DISCUSSION

The information gained suggests that all three modified nucleoside 5′-triphosphates with a flattened ribose

Fig. 3. PAGE separation of the products of primer elongation catalyzed by DNA polymerase I. Tracks 1,2: synthesis in the absence of termination substrates (control). Tracks 3–6: synthesis in the presence of ddATP (3), ddGTP (4), ddCTP (5), and ddTTP (6), respectively. Tracks 7–10: in the presence of ltTTP at the following concentrations: 300 μM (7), 600 μM (8), 900 μM (9), and 1.8 mM (10). Assays 2–10 were subsequently chased with an excess of four dNTPs.

Table III

K_m values (μM) and V_{max}/V_{max}(dTTP) ratios for ltTTP, rtTTP, laTTP, and dTTP in the DNA synthesis reaction catalysed by AMV reverse transcriptase

Compound	K_m	V_{max}/V_{max}(dTTP)
dTTP	1.25	1
ltTTP	2.7	0.3
rtTTP	2.2	0.38
laTTP	3.1	0.47

ring structure are termination substrates of a series of DNA polymerases. These findings favour the assumption that modified substrates with a flattened ribose cycle structure imitate the transition state conformation of the substrates in The [DNA polymerase–template–primer] complex. Modified substrates of this type were shown to inhibit HIV reverse transcriptase most effectively. This observation is in agreement with the concept of a lesser substrate specificity of reverse transcriptases.

The K_m and V_{max} values for modified substrates and dTTP are shown to differ only 2- to 3-fold. These results indicate that modified substrates with a flattened ribose ring correspond to the DNA polymerases active site structure.

It has been suggested [21] that at the first stage of nucleotide residue incorporation into the DNA chain, the ribose cycle locates the nucleic base correctly relative to the 5′-hydroxyl group. Starting from this suggestion we might reason that the flattened glycone structure meets the requirements for this function.

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