Interaction of polymerases with 2′-deoxyuridine-5′-triphosphate spin-labeled at the 5-position

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2′-Deoxyuridine-5′-triphosphate spin-labeled at the 5-position with *N*-[1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl]-O- was found to be an inhibitor of some DNA and RNA polymerases including avian myeloblastosis virus reverse transcriptase. Furthermore, the spin-labeled nucleotide was found to be incorporated internally into polydeoxythymidylic acid via reverse transcriptase to an extent of 1.0 spin-labeled base per 10^3 bases. The incorporation, monitored by electron spin resonance, is analogous to some other nucleotide inhibitors of polymerases, and the results indicate that it may be feasible to obtain sequence specific, spin-labeled DNA, enzymatically.

Spin-labeled nucleotide *Reverse transcriptase inhibition* *DNA polymerase*

1. INTRODUCTION

It has been proposed that inhibition of various DNA polymerases by some nucleotide analogs such as F_3dTTP and araCTP is due to the impedance of polymerization, i.e., formation of the phosphodiester bond between araCTP or F_3dTTP and the 3′-hydroxyl of the primer is slow, since both nucleotide analogs can be incorporated internally into DNA [1–14]. The ability of araCTP to impede polymerization has been exploited to investigate the mechanism of DNA replication in calf liver cells. These studies show that initiation of nascent DNA synthesis can occur at some distance from the replication fork since new Okazaki fragments accumulate in the presence of araCTP [12]. Thus, the inhibitory properties of some nucleotide analogs can be utilized to study biological processes such as replication.

Abbreviations: F_3dTTP, 5-trifluoromethyl-2′-deoxyuridine-5′-triphosphate; araCTP, cytosine-D-arabinofuranoside-5′-triphosphate; pppDUGT, 2′-deoxyuridine-5′-triphosphate substituted at the 5-position with *N*-[1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl]-0-; AMV, avian myeloblastosis virus; ESR, electron spin resonance

The present work was undertaken to investigate: (1) the inhibitory properties of pppDUGT, a dUTP analog substituted at the 5-position with *N*-[1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl]-0-, toward AMV reverse transcriptase as well as other DNA or RNA polymerases; (2) the incorporation of pppDUGT via AMV reverse transcriptase into DNA which can be monitored by ESR. These studies were done to relate the inhibitory properties of pppDUGT to those of other nucleotide analogs and to explore the possibility of using a DNA polymerase such as reverse transcriptase to obtain sequence specific, spin-labeled DNA. Sequence nonspecific, spin-labeled DNAs and RNAs, obtained by chemical alkylation or enzymatic copolymerization of spin-labeled and unlabeled nucleotides [15–17], have been used in this laboratory to investigate various nucleic acid–nucleic acid and nucleic acid–protein interactions [18–21].

2. MATERIALS AND METHODS

2.1. *Materials*

Oligo $(dG)_{12-18}$ and oligo $(dT)_{12-18}$ were obtained from Collaborative Research and $(C)_n$ and $(A)_n$ from Miles Laboratories. DeoxyUTP,

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oh^{5}dUTP, and unlabeled dNTPs were obtained from Sigma Chemical Co. [^{3}H]dGTP (spec. act. = 6.1 Ci/mmol) and [^{3}H]dTTP (spec. act. = 18.5 Ci/mmol) were purchased from New England Nuclear. DE81 filters (2.4 cm) were obtained from Whatman. DUGT was synthesized according to a published procedure [22] and pppDUGT according to Bobst [23].

2.2. *Enzymes*

Purified, homogeneous AMV reverse transcriptase was generously provided by Dr Joseph Beard (Life Sciences, Inc.) and was stored at −20°C in 50% glycerol, 0.2 M potassium phosphate buffer (pH 7.2), 2 mM dithiothreitol, and 0.2% Triton X-100. Novikoff hepatoma DNA polymerase β (Fraction VI) was purified to homogeneity according to Stalker et al. [24]. Novikoff hepatoma DNA polymerase α (Fraction V) was purified according to Rein and Meyer (unpublished results). *E. coli* DNA polymerase I was purified to homogeneity according to Slater et al. [25]. DNA polymerase III holoenzyme (Fraction V) was purified according to McHenry and Kornberg [26]. A published procedure was used to obtain purified influenza virus ribonucleoprotein–RNA polymerase complex isolated from the A_o/WSN strain of influenza virus [27]. Highly purified, nuclease free RNA polymerase from *E. coli* was obtained from P-L Biochemicals, Inc.

2.3. *Enzyme assays*

AMV reverse transcriptase activity was measured in a volume of 100 μl containing: 50 mM Tris–HCl buffer (pH 8.3), 40 mM KCl, 6 mM $MgCl_2$, 0.15 mM [^{3}H]dGTP or 0.12 mM [^{3}H]dTTP (spec. act. = 5–25 cpm/pmol), and 2.5 μg/ml $(C)_n \cdot (dG)_{12-18}$ (6:1) or 2.7 μg/ml $(A)_n \cdot (dT)_{12-18}$ (6:1). After addition of enzyme, the reaction was allowed to proceed for 30 min at 37°C and was terminated by spotting 50 μl of reaction mixture on Whatman DE81 filters. The filters were washed extensively with 0.19 M K_2HPO_4, distilled water, and 95% ethanol, respectively. The filters were dried and counted using toluene based scintillation fluid.

Novikoff hepatoma DNA polymerases α and β and *E. coli* DNA polymerase I were assayed in a total volume of 25 μl containing: 25 mM Tris–HCl buffer (pH 8.4), 5 mM 2-mercaptoethanol, 7 mM magnesium acetate, 0.5 mM EDTA, 50 mM NaCl, 0.015 mM each dTTP, dCTP, dGTP, and [^{3}H]dATP (spec. act. = 325 mCi/mmol), 15% (w/v) glycerol, 100 μg/ml activated DNA and 0.01 to 0.3 units of enzyme. The reactions were incubated for 30 min at 37°C and terminated with the addition of 0.5 ml of cold 10% trichloroacetic acid and 20 mM sodium pyrophosphate. The incorporation of [^{3}H]dATP into acid insoluble product was determined on filter paper discs as previously described [28].

E. coli DNA polymerase III holoenzyme was assayed with G4 DNA as the template in the presence of single stranded binding (SSB) and dnaG proteins [29].

Influenza virus RNA polymerase activity was measured in a total volume of 50 μl containing: 67 mM Tris–HCl buffer (pH 8.1), 0.83 mM CTP, 0.83 mM GTP, 1.66 mM ATP, 0.15 mM [^{32}P]UTP (spec. act. = 604 cpm/pmol), 33.3 mM ammonium sulfate, 0.67 mM dithiothreitol, 8 mM $MgCl_2$, 0.4 mM AG (adenylyl-3′ ⟶ 5′-guanoine), and viral transcriptase complex and inorganic pyrophosphatase (0.3 units). The reactions were incubated at 31°C for 60 min and terminated by addition of 0.1 ml of 0.1 M sodium pyrophosphate, 0.1 ml of cold 0.5 mM UTP, 0.2 ml carrier RNA, bovine serum albumin, and 1 ml of 10% trichloroacetic acid. After 60 min on ice the precipitated material was collected on Whatman GF/A filters and washed with 60 ml of 6% trichloroacetic acid containing 50 mM sodium pyrophosphate followed by 20 ml of 1% trichloroacetic acid. The filters were dried, acid neutralized by the addition of 0.5 ml of 10 mM NaOH and counted using 5 ml of Bray's scintillation cocktail.

E. coli RNA polymerase activity was measured in a total volume of 100 μl containing: 32 mM Tris–HCl buffer (pH 7.9), 8 mM $MgCl_2$, 0.08 mM dithiothreitol, 0.12 M KCl, and 0.08 mM EDTA; 0.23 mM each of CTP, UTP, and GTP and [^{3}H]ATP (spec. act. = 9 cpm/pmol); 0.17 mg/ml activated calf thymus DNA; 0.3 mg/ml bovine serum albumin; and 4 μg RNA polymerase. After preincubation of all components except the polymerase at 37°C for 10 min, the polymerase was added, and the reaction was allowed to proceed for 10 min. The reaction was terminated by spotting 50 μl on a Whatman DE81 filter. The filters were

washed and counted by the same procedure used for reverse transcriptase.

2.4. *Inhibition studies*

Inhibition of reverse transcriptase activity was studied either (a) by addition of inhibitor to a 100 μl enzyme solution containing 50 mM Tris–HCl (pH 8.3), 40 mM KCl, and 6 mM $MgCl_2$ and removal of 5 μl aliquots at various time intervals for assay; or (b) addition of enzyme to a standard assay containing inhibitor. Inhibition is expressed as residual activity, $v_i/v_c \times 100$ (%), where v_i = activity in the presence of inhibitor and v_c = activity in the absence of inhibitor.

2.5. *Incorporation of pppDUGT into (DUGT,dT)$_n$ via reverse transcriptase*

The polymerization reaction was done in a volume of 1.0 ml in 0.05 M Tris–HCl buffer (pH 8.3) containing 0.12 M KCl, 10 mM $MgCl_2$, 0.3 mM dithiothreitol, 280 μg/ml $(A)_n \cdot (dT)_{12-18}$ (6:1), 0.5 mM dTTP, 0.05 mM pppDUGT, and 2850 units/ml of reverse transcriptase. The reaction was initiated by the addition of enzyme and allowed to proceed for 90 min at 37°C. The reaction was terminated and protein extracted with chloroform/isoamyl alcohol (5:2) followed by centrifugation. The extraction was repeated three times followed by addition of 0.1 ml of 3 M NaOH. The reaction was incubated for 1 h at 60°C and then acid neutralized with 0.1 ml of 3 M HCl. The polymers were purified by chromatography on a Sephacryl S-200 column eluting with 0.04 M NH_4HCO_3.

The incorporation of spin label into the polymer was measured after concentrating the high molecular weight material eluted from Sephacryl S-200 by lyophilization. The concentration of spin label was measured by ESR by comparing the h_o peak of the ESR spectrum of (DUGT,dT)$_n$ synthesized with reverse transcriptase with the h_o peak of the spectrum of (DUGT,dT)$_n$ prepared by an alternative method containing a known concentration of spin label [23]. The concentration of (dT)$_n$ or (DUGT,dT)$_n$ was determined using ϵ_{267} = 8540 M^{-1} cm^{-1}.

3. RESULTS

3.1. *Effect of pppDUGT on various polymerases*

Inhibition of DNA and RNA polymerases from various sources by 0.5 mM pppDUGT was compared. The results in table 1 show that influenza virus RNA polymerase was least inhibited by pppDUGT while the most pronounced inhibition was observed with AMV reverse transcriptase and DNA polymerase α from Novikoff hepatoma cells.

3.2. *Inhibition of AMV reverse transcriptase by pppDUGT and its analogs*

As shown in table 2, increasing inhibition of

Table 1

Effect of 0.5 mM pppDUGT on DNA or RNA polymerases from various sources

Polymerase[a]	v_i/v_c[b] × 100(%)
Novikoff hepatoma	
DNA pol α	14
DNA pol β	28
E. coli	
DNA pol I	53
DNA pol III holoenzyme	32
RNA polymerase	29
Influenza virus	
RNA pol complex	80
AMV reverse transcriptase	14

[a] The assay procedures for the polymerases are described in section 2

[b] v_i = polymerase activity in the presence of the inhibitor and v_c = polymerase activity in the absence of inhibitor

Table 2

Inhibition of AMV reverse transcriptase by pppDUGT and its analogs

Inhibitor	I (mM)	$v_i/v_c \times 100$ (%)	Template · primer
pppDUGT	1.0	14	$(C)_n \cdot (dG)_{12-18}$
	0.1	73	$(C)_n \cdot (dG)_{12-18}$
	0.1	69	$(A)_n \cdot (dT)_{12-18}$
	0.01	90	$(C)_n \cdot (dG)_{12-18}$
	0.01	90	$(A)_n \cdot (dT)_{12-18}$
DUGT	1.1	92	$(C)_n \cdot (dG)_{12-18}$
dUTP	1.1	121	$(C)_n \cdot (dG)_{11-18}$
oh^{5}dUTP	1.1	106	$(C)_n \cdot (dG)_{12-18}$

AMV reverse transcriptase was observed with increasing pppDUGT concentrations regardless of the template·primer used. The effect of the pppDUGT analogs, oh^{5}dUTP and DUGT, on reverse transcriptase activity was studied, and as a control the effect of dUTP, a polymerase substrate, was also observed. The results in table 2 show that while reverse transcriptase activity was strongly inhibited by 1 mM pppDUGT, oh^{5}dUTP and DUGT have no effect on activity, and the activity in the presence of dUTP is actually slightly greater than in its absence which has also been observed for DNA polymerase I using d(A-T)$_n$ in the presence or absence of dUTP [30]. Therefore, the observed inhibition of reverse transcriptase requires both the spin label and triphosphate moieties.

It was previously shown that AMV reverse transcriptase contains a sensitive thiol group(s) at the active site of the enzyme which can be protected from inactivation by *N*-ethylmaleimide by preincubating the enzyme with template·primer [31]. To eliminate the possibility that pppDUGT inhibits reverse transcriptase through oxidation of this sensitive thiol group(s) by the nitroxide radical, inhibition by pppDUGT was observed after preincubating the enzyme with template·primer or dNTP. The enzyme was preincubated with or without $(C)_n \cdot (dG)_{12-18}$, $(A)_n \cdot (dT)_{12-18}$, dGTP, or dTTP, 1 mM pppDUGT was added, and 5 μl aliquots were assayed at various time intervals. Also, the enzyme was preincubated with or without substrate, and 1 mM pppDUGT was added with the other components necessary for a complete assay mixture. In all instances the inhibition was similar to that shown in table 2 indicating that pppDUGT does not oxidize any essential thiol groups of the enzyme.

3.3. *Kinetics of inhibition of reverse transcriptase by pppDUGT*

The kinetics of inhibition for pppDUGT were determined, treating either dTTP or $(A)_n \cdot (dT)_{12-18}$ as the variable substrate. In figs.1A and B, Lineweaver–Burke plots of the data show

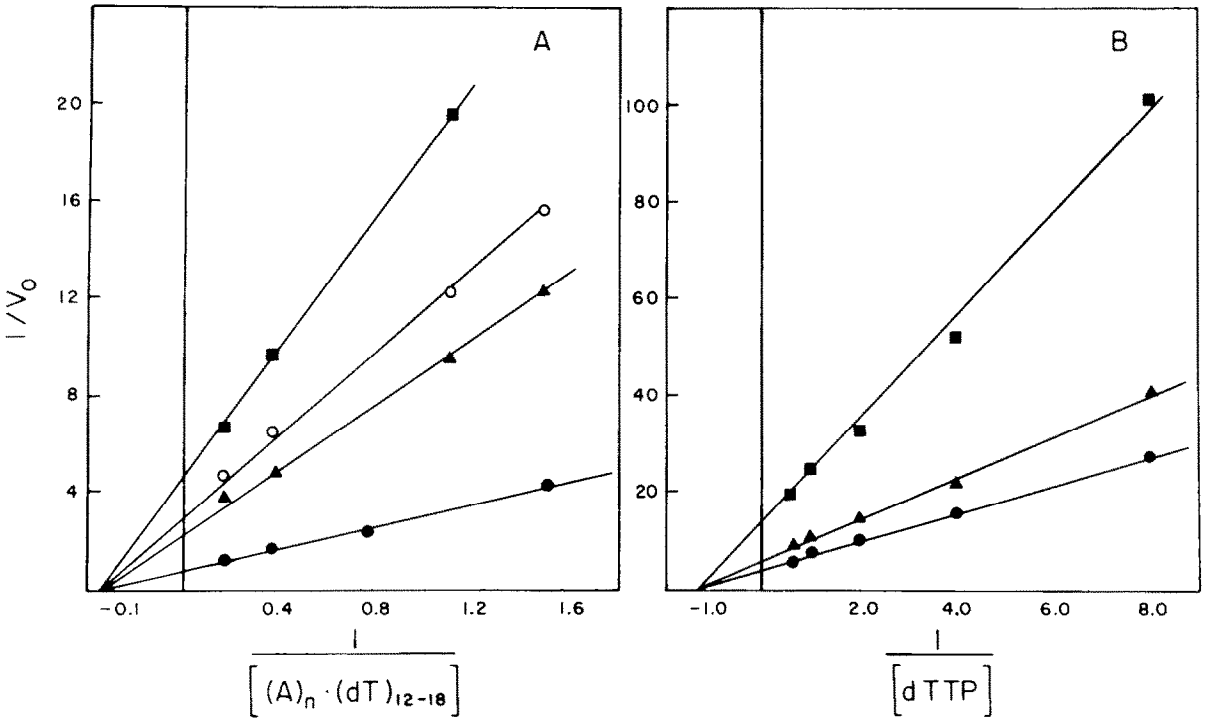

Fig.1. Kinetics of inhibition of AMV DNA polymerase activity using the following concentrations of pppDUGT: (■) 1.12×10^{-5} M; (○) 5.60×10^{-5} M; (▲) 1.12×10^{-4} M; (●) none. In experiment A, $(A)_n \cdot (dT)_{12-18}$ (6:1) was the variable substrate from 0.46 to 5.46 μg/ml. In experiment B, dTTP was the variable substrate from 1.0×10^{-5} to 1.25×10^{-4} M.

that pppDUGT inhibits reverse transcriptase activity noncompetitively or mixed with respect to either dTTP or $(A)_n \cdot (dT)_{12-18}$. The apparent K_m values obtained from the data in figs.1A and B for dTTP and $(A)_n \cdot (dT)_{12-18}$ are 77 μM and 14 μg/ml, respectively. The K_i calculated from the data for pppDUGT with dTTP as the variable substrate was 8.0 μM determined from the equation: $K_i = i/(v_i/v_p)-1$, where i = [pppDUGT], v_i = maximal velocity of the control, and v_p = maximal velocity of the inhibited reaction [32].

3.4. *Incorporation of pppDUGT into (DUGT,dT)$_n$ and the effect of pppDUGT on the size of the cDNA product*

Incorporation of pppDUGT into cDNA via reverse transcriptase was investigated using $(A)_n \cdot (dT)_{12-18}$ (6:1) with a dTTP/pppDUGT ratio of 10:1. The polymerization reaction was also carried out in the absence of pppDUGT to serve as a control. A comparison was made of the elution profiles obtained from chromatographing the polymerization reaction, after extraction, on Sephacryl S-200 in the presence or absence of pppDUGT to determine the effect of pppDUGT on the product size. The elution profiles for $(dT)_n$ or $(DUGT,dT)_n$ were identical, i.e., no low molecular weight polymer was observed in the presence of pppDUGT, indicating that pppDUGT does not act as a chain terminator.

The ratio of pppDUGT/dTTP incorporation into $(DUGT,dT)_n$, measured by ESR as described in section 2, is 1:1000. Two procedures were used to ascertain that the spin label was incorporated into the copolymer. First, the h_{-1}/h_0 ratio of the ESR spectrum of the spin labeled polymer was measured and compared to h_{-1}/h_0 of $(DUGT,dT)_n$ prepared by an alternative procedure. Our results have shown that the h_{-1}/h_0 ratio decreases significantly from 0.80 ± 0.02 for pppDUGT to 0.60 ± 0.02 when DUGT is incorporated into $(DUGT,dT)_n$ [23]. The h_{-1}/h_0 of the spin-labeled material obtained here was 0.60 indicating that the spin label is incorporated into the polymer. Secondly, as shown in fig.2, the spin-labeled polymer was subjected to a test for free spin label which has been previously described [16]. When poly L-lysine was added to the spin-labeled polymer, the ESR spectrum of the spin label markedly broadened which is observed when a spin label is incorporated into a polynucleotide. As a control poly (L-lys) was added to a mixture of dTTP and pppDUGT at approximately the same ratio of dTTP/pppDUGT found in the copolymer. As shown in fig.2 the ESR spectrum remains essentially unchanged except for a small dilution factor. These results conclusively demonstrate that the spin label is incorporated into $(DUGT,dT)_n$.

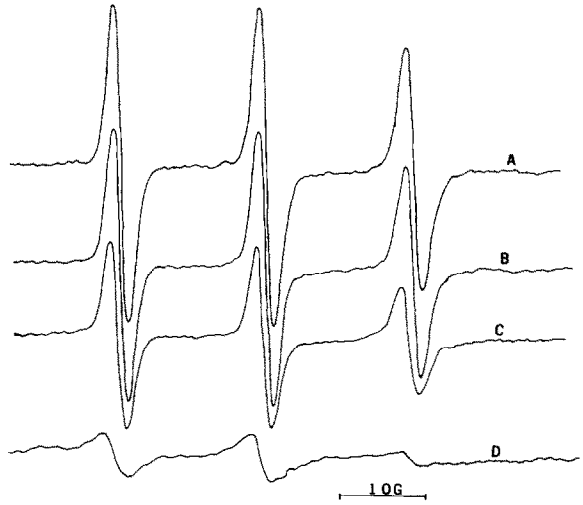

Fig.2. Poly L-lysine test demonstrating the incorporation of pppDUGT into $(DUGT,dT)_n$. (A) ESR spectrum of 1.47 mM dTTP and 1.50 μM pppDUGT in 0.10 M Tris–HCl buffer (pH 7.0) containing 0.05 M KCl; (B) same as A plus 2.87 mM poly L-lysine. (C) ESR spectrum of 2.05 mM $(DUGT,dT)_n$ in same buffer as (A) obtained from polymerization of pppDUGT and dTTP (ratio 1:10) by reverse transcriptase; (D) same as (C) plus 3.9 mM poly L-lysine.

4. DISCUSSION

Several DNA polymerases of mammalian, bacterial, or viral origin as well as *E. coli* RNA polymerase were inhibited by pppDUGT while influenza virus RNA polymerase was inhibited only slightly. The pppDUGT analogs, DUGT and oh^{5}dUTP, have no effect on reverse transcriptase activity indicating that both the triphosphate and spin label moieties are required for inhibition. The noncompetitive or mixed inhibition kinetics observed with pppDUGT using dTTP as the variable substrate, while somewhat unexpected, is similar to the inhibition kinetics of HeLa cell or vaccinia virus induced DNA polymerases by F_3dTTP with dCTP or dATP as variable substrates [3].

Incorporation of pppDUGT into $(DUGT,dT)_n$ via reverse transcriptase occurs to the extent of 1.0 pDUGT residue/10^3 pdT residues when $(A)_n \cdot (dT)_{12-18}$ is used as template·primer and the pppDUGT/dTTP ratio is 1:10 in the reaction mixture. It is interesting to note that the rate of incorporation of pppDUGT into $(DUGT,dT)_n$ is similar to the misincorporation rate of one mismatched base/600–700 residues previously observed for AMV reverse transcriptase using $(A)_n \cdot (dT)_{12-18}$ and dTTP and dCTP at a ratio of 5:2 [33].

The results show that inhibition of reverse transcriptase by pppDUGT is due to a decreased rate of polymerization with limited, internal incorporation of pDUGT into DNA similar to araCTP and F_3dTTP inhibition of DNA polymerases from various sources. The internal incorporation of pppDUGT into $(DUGT,dT)_n$ is supported by the gel filtration elution profile of $(DUGT,dT)_n$ and by the ESR lineshape of $(DUGT,dT)_n$. The results indicate that pppDUGT may be useful for studying DNA replication since it impedes polymerization, or as a reporter group in biological systems. Furthermore, it appears feasible to obtain sequence specific, spin-labeled DNA which would be very useful for studies on various biochemical events where nucleic acid interactions occur.

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