Biochemical Pharmacology, Vol. 29, pp. 1149–1153.

0006-2952/80/0415-1149 $02.00/0

INHIBITION OF DEOXYRIBONUCLEIC ACID POLYMERASES OF HUMAN LEUKEMIC LEUKOCYTES BY 2′,3′-DIDEOXYTHYMIDINE TRIPHOSPHATE*

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(*Received* 30 *August* 1979; *accepted* 26 *October* 1979)

Abstract—The effects of 2′,3′-dideoxythymidine 5′-triphosphate (d_2TTP) on the activities of DNA polymerases α and β isolated from leukocytes of a patient with acute myelogenous leukemia have been examined. DNA polymerase β was more sensitive than DNA polymerase α to d_2TTP inhibition; the concentration of d_2TTP required for a 50 per cent inhibition of enzyme activity was 20-fold lower for DNA polymerase β than for DNA polymerase α in assays with activated DNA template. A similar difference in sensitivity was observed also when synthetic primer templates such as (dT) $\sim_{15}$·$(dA)_n$ or (dT) $\sim_{15}$·$(A)_n$ were used. However, with (dG) $\sim_{15}$·$(dC)_n$ primer template, neither DNA polymerase α nor DNA polymerase β activity was inhibited by d_2TTP. Kinetic analysis with activated DNA template showed that the d_2TTP inhibition was competitive with dTTP but noncompetitive with dGTP; the values of the K_m for dTTP and K_i for d_2TTP for the DNA polymerase α were approximately the same, while for DNA polymerase β the K_m was twenty times more than K_i. Neither DNA polymerase α nor DNA polymerase β had any exonuclease activity and, therefore, the resistance of DNA polymerase α to d_2TTP inhibition was not due to exonuclease activity. The extent of d_2TTP inhibition was not altered significantly by changes in the concentration of either the template or the enzyme. Preincubation of the inhibitor with either the template or the enzyme was not necessary for inhibition. The compound was inhibitory even when added after the initiation of the reaction.

The existence of multiple species of DNA polymerases in dividing mammalian cells has been well documented [1–3]. The biological role of the individual polymerases, however, has not yet been unequivocally established. One of the approaches to determine the specific roles that these polymerases play in DNA replication is to examine whether compounds that inhibit cellular DNA synthesis have a preferential effect on any of the polymerase species. A number of deoxynucleotide analogs compete with normal substrates, are incorporated into DNA *in vitro*, and effectively terminate chain growth (reviewed in Ref. 4). An analog of deoxythymidine triphosphate, 2′,3′-dideoxythymidine 5′-triphosphate (d_2TTP), has been used as a reagent to control and analyze bacterial DNA replication *in vitro* [5]. The d_2TTP has the 3′-hydroxyl group of 2′-deoxyribothymidine triphosphate replaced by a hydrogen. This compound blocks chain growth during bacterial DNA replication by preventing the attachment of additional nucleotide residues. The ability of d_2TTP to block 3′ ends has been utilized in characterizing multiple catalytic functions of bacterial DNA polymerases. Studies with purified *Escherichia coli* DNA polymerase I showed that d_2TTP inhibited polymerization and related functions such as pyrophosphate exchange, pyrophosphorolysis and hydrolysis at the 3′-terminus but did not interrupt 5′→3′ hydrolysis [5,6]. Besides inhibiting DNA synthesis by *E. coli* DNA polymerase I [5], d_2TTP also inhibits the reverse transcriptase of Rous sarcoma virus [7] and avian myeloblastosis virus [8]. The DNA synthesized in the presence of d_2TTP is of short chain length [7]. Recently, use of the dideoxynucleotide analogs in determining the nucleotide sequence of DNA has been described [9].

Although the detailed mechanism of d_2TTP inhibition was studied with bacterial DNA polymerase, the mechanism of inhibition of mammalian DNA polymerases has not been reported. We compared the effect of d_2TTP on the activities of DNA polymerases α and β isolated from leukocytes of a patient with an acute myelogenous leukemia (AML) and found that DNA polymerase β was more sensitive than DNA polymerase α to d_2TTP inhibition. We report here a comparison of inhibition by this nucleotide analog of DNA polymerases from human leukemia cells and a detailed mechanism of the inhibition. During the course of our study, Edenberg *et al.* [10] and Waqar *et al.* [11] reported that DNA polymerase β was more sensitive than DNA polymerase α to d_2TTP inhibition.

MATERIALS AND METHODS

Tritiated deoxynucleoside triphosphates and Triton X-100 were obtained from the New England Nuclear Corp., Boston, MA.; d_2TTP, nucleoside triphosphates and synthetic primer templates were purchased from P. L. Biochemicals, Milwaukee, WI. The oligonucleotide primers contained 12–18 nucleotides. Calf thymus DNA was converted to the activated form by treatment with DNase I, according to the procedure of Schlabach *et al.* [12].

Isolation of cellular DNA polymerases. The cellular DNA polymerases α and β were isolated from

*This research was supported by Grant CH-47A of the American Cancer Society and CA-08341 of the United States Public Health Service.

leukocytes of an AML patient. The details of the isolation procedure have been described elsewhere (Ref. 13, also in H. S. Allaudeen, manuscript in preparation). Essentially, the nucleic acids were removed from the total cell extract by passage through a DEAE cellulose column and elution with a 0.3M KCl containing buffer. The DNA polymerases α and β were clearly separated from each other upon chromatography on a phosphocellulose column. The peak fractions of the DNA polymerase α and the DNA polymerase β which eluted at 0.19 M and 0.4 M KCl concentrations, respectively, were used for the study. The DNA polymerase α and the DNA polymerase β resembled the respective polymerases of other mammalian cells in many of their catalytic and structural properties [1–3].

Enzyme assays. DNA polymerase α activity was assayed in a 50 μl reaction mixture which contained 50 mM Tris–HCl (pH 7.5), 2 mM dithiothreitol, 8 mM $MgCl_2$, 100 μM each of dATP, dCTP and dGTP, and 40 μM [^{3}H]dTTP (450 c.p.m./pmole), 10 μg of activated calf thymus DNA, 10–20 μg of bovine serum albumin, 5–10% glycerol, and enzyme. Incubation was at 37° for 30 min. Acid-insoluble radioactivity was collected on a nitrocellulose filter (Gelman or Amicon), washed several times with 5% trichloroacetic acid containing 2 mM sodium pyrophosphate, once with 70% ethanol, dried, and measured in a liquid scintillation counter.

DNA polymerase β activity was assayed under similar conditions except that a pH 8.5 Tris–HCl buffer and 40 mM KCl were used.

When synthetic primer templates such as (dT) $_{\sim 15}$·(dA)$_n$ or (dT) $_{\sim 15}$·(A)$_n$ were used, the conditions were similar except for the following changes: 1 μg of the primer template, 80–160 μM [^{3}H]dTTP (225 c.p.m./pmole), 40 mM NaCl, and either 4 mM $MgCl_2$ for (dT) $_{\sim 15}$·(dA)$_n$ or 1 mM $MnCl_2$ for (dT) $_{\sim 15}$·(A)$_n$. With (dG) $_{\sim 15}$·(dC)$_n$ primer template, 40 μM [^{3}H]dGTP (450 c.p.m./pmole), and 4 mM $MgCl_2$ were used.

RESULTS

Inhibition of DNA polymerase α and β activities by d_2TTP. The effects of d_2TTP on the activities of DNA polymerases α and β were examined using activated DNA as the template and [^{3}H]dTTP as the rate-limiting substrate. Addition of d_2TTP to the reaction mixture inhibited the activities of both enzymes (Fig. 1). However, the DNA polymerase β was more sensitive than the DNA polymerase α to d_2TTP inhibition; for example, the amount of d_2TTP required for a 50 per cent inhibition of activity was 20-fold higher for DNA polymerase α than that required for DNA polymerase β. We noticed a similar difference in sensitivity when the [^{3}H]dTTP was replaced with [^{3}H]dGTP as the rate-limiting substrate. However, in this case, twice the amount of d_2TTP was required for a 50 per cent inhibition of the DNA polymerase β activity.

Exonuclease activity and mixing experiments. In order to determine whether the difference between DNA polymerase α and DNA polymerase β in their sensitivities to d_2TTP inhibition was due to exonuclease activity associated with DNA polymerase α,

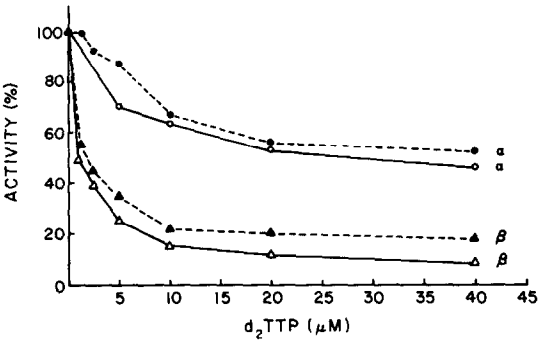

Fig. 1. d_2TTp inhibition of DNA polymerase α and DNA polymerase β. Percentage of remaining activity with increasing concentrations of d_2TTP is shown. Either [^{3}H]dTTP or [^{3}H]dGTP was the rate-limiting substrate in assays with activated DNA template. Key: DNA polymerase α with [^{3}H]dTTP (○———○), DNA polymerase β with [^{3}H]dTTP (△———△), DNA polymerase α with [^{3}H]dGTP (● - - ●), and DNA polymerase β with [^{3}H]dGTP (▲ - - ▲). In reactions with either [^{3}H]dTTP or [^{3}H]dGTP as the rate-limiting substrate (20 μM for the DNA polymerase α and 40 μM for the DNA polymerase β), the other three deoxynucleoside triphosphates were maintained at 100 μM. One hundred per cent activity of DNA polymerase α and DNA polymerase β represents 32–39 pmoles of [^{3}H]dNMP incorporation. Specific activities of the DNA polymerase α and DNA polymerase β are 17.9 and 15.4 units, respectively. One unit of enzyme activity represents 1 nmole of [^{3}H]dNMP incorporated with activated DNA template per mg of protein per hr. Other assay conditions are described in Materials and Methods.

we performed two experiments. First, the DNA polymerase α and DNA polymerase β preparations were tested for any exonuclease activity using *in vitro* labeled [^{32}P]DNA. Neither enzyme contained any detectable exonuclease activity. Second, known amounts of DNA polymerase α and DNA polymerase β were mixed together and the total DNA polymerase activity was determined in the presence and absence of d_2TTP; there was no significant difference in the extent of d_2TTp inhibition. These results indicate that the resistance of DNA polymerase α to d_2TTP inhibition was not due to exonuclease present in preparations of DNA polymerase α.

Time course of d_2TTP inhibition. To determine whether d_2TTP could inhibit DNA synthesis even after the reaction was initiated, d_2TTP was added to the ongoing reaction mixture at different times, and the activity was monitored. The compound caused an instantaneous inhibition whether the drug was added at the beginning or after the initiation of the reaction (Fig. 2).

d_2TTP inhibition with different primer templates. We compared the effect of d_2TTP on the activities of the DNA polymerases with different primer templates in addition to the activated DNA. Synthetic primer templates such as (dT) $_{\sim 15}$·(dA)$_n$, (dT) $_{\sim 15}$·(A)$_n$ and (dG) $_{\sim 15}$·(dC)$_n$ were used under appropriate assay conditions (Table 1 and Fig. 3).

The effects of d_2TTP on the activities of DNA polymerases α and β with (dT) $_{\sim 15}$·(dA)$_n$ primer template are shown in Fig. 3; its effect on the DNA polymerase β activity with (dT) $_{\sim 15}$·(A)$_n$ is also

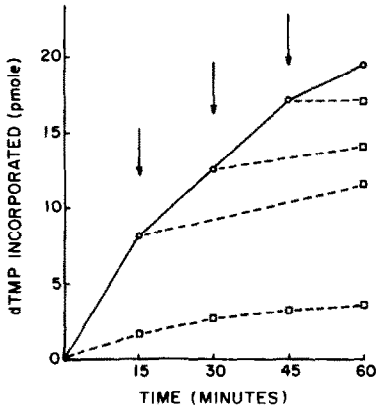

Fig. 2. Time course of d_2TTP inhibition of the DNA polymerase β. d_2TTP at a final concentration of 5×10^{-6} M was added at the time interval shown after the initiation of the enzyme reaction with [^{3}H]dTTP as the rate-limiting substrate and activated DNA template; all other conditions are described in the text.

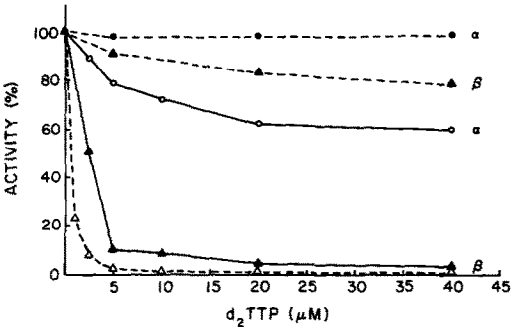

Fig. 3. d_2TTP inhibition with different synthetic primer templates. Key: (dT)$_{\sim 15}$·(dA)$_n$ primer template with DNA polymerase α (○———○), and DNA polymerase β (▲———▲); (dT)$_{\sim 15}$·(A)$_n$ primer template with DNA polymerase β (△———△); (dG)$_{\sim 15}$·(dC)$_n$ primer template with DNA polymerase α (● – – ●), and DNA polymerase β (▲ – – ▲). One hundred per cent of DNA polymerase α and DNA polymerase β activities ranged from 19.2 to 22.7 pmoles of the respective [^{3}H]dNMP incorporation.

shown in the figure. The d_2TTP inhibition of DNA polymerase β activity was maximum with (dT)$_{\sim 15}$·(A)$_n$ primer template. However, d_2TTP did not significantly inhibit the activity of either DNA polymerase α or DNA polymerase β when (dG)$_{\sim 15}$·(dC)$_n$ primer template was used (Fig. 3). For instance, 5 μM d_2TTP inhibited 97 per cent of the DNA polymerase β activity with (dT)$_{\sim 15}$·(A)$_n$ primer template, whereas the same amount of d_2TTp inhibited only 9 per cent of the enzyme activity with (dG)$_{\sim 15}$·(dC)$_n$ primer template.

Kinetics of d_2TTP inhibition. To characterize further the nature of the inhibition, we examined inhibition by d_2TTP, with increasing substrate concentrations. In assays with activated DNA template, tritiated dTTP was used as the rate-limiting substrate and the other three triphosphates were in excess. When the data were plotted by the method of Lineweaver and Burk [14], straight lines could be drawn intersecting on the ordinate, indicating that the inhibition was competitive with dTTP (Fig. 4). The apparent K_m value of DNA polymerase α for [^{3}H]dTTP was 10 μM; the apparent K_i for d_2TTP was similar (9.6 μM). A similar pattern of inhibition was observed with DNA polymerase β; however, lower concentrations of d_2TTP were required for the inhibition (Fig 5). The apparent K_m value of DNA

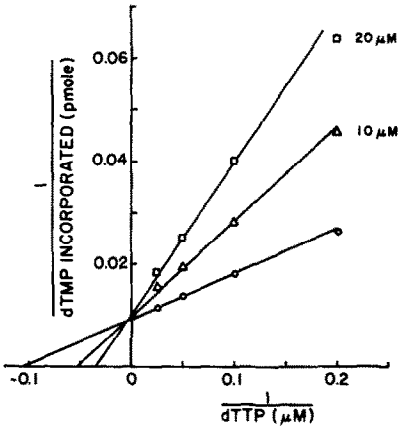

Fig. 4. Effect of d_2TTP on the reaction rate in the presence of different concentrations of [^{3}H]dTTP with activated DNA template of DNA polymerase α.

polymerase β for [^{3}H]dTTP was 21 μM; the K_i for d_2TTP was approximately twenty times lower than the K_m (1.1 μM). When similar experiments were performed with DNA polymerase β using [^{3}H]dGTP as the rate-limiting substrate, the straight lines inter-

Table 1. Inhibition by d_2TTP with different primer templates*

Primer template	[^{3}H]dNMP incorporated	d_2TTP (μM)	DNA polymerase α inhibition (%)	DNA polymerase β inhibition (%)
Activated DNA	dTMP	1.0	3.5	52.0
Activated DNA	dGMP	1.0	1.5	35.0
(dT)$_{\sim 15}$·(dA)$_n$	dTMP	1.0	3.2	21.0
(dT)$_{\sim 15}$·(A)$_n$	dTMP	1.0		77.5
(dG)$_{\sim 15}$·(dC)$_n$	dGMP	5.0	2	9.0

* Enzyme activities were determined using assay conditions optimum for the respective enzymes with each primer template.

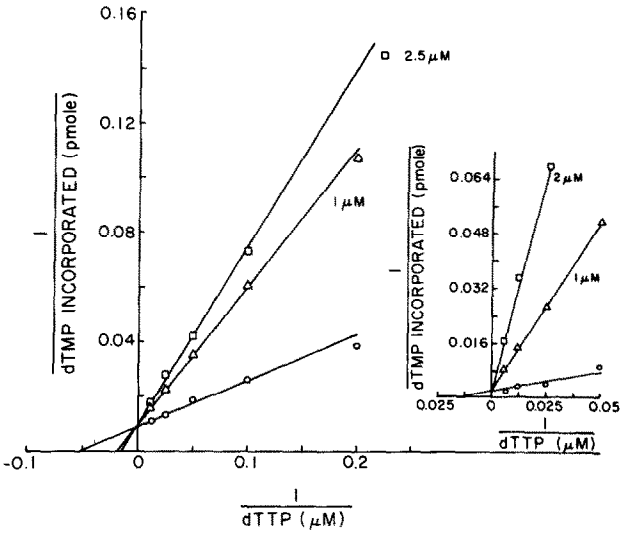

Fig. 5. Effect of d_2TTP on the reaction in the presence of different concentrations of [^{3}H]dTTP with activated DNA template of DNA polymerase β. Inset: effect of d_2TTP on the reaction rate of DNA polymerase β in the presence of different concentrations of [^{3}H]dTTP with $(dT)_{\sim 15}\cdot(A)_n$ primer template.

sected on the abscissa (data not presented). These plots indicate that the inhibition was non-competitive with dGTP. The apparent K_m value of DNA polymerase β for dGTP was 6 μM; the K_i for d_2TTP was approximately 1.7 μM. The d_2TTP inhibition of DNA polymerase β activity was also examined with increasing concentrations of [^{3}H]dTTP using $(dT)_{\sim 15}\cdot(A)_n$ primer template. The Lineweaver–Burk plot indicates that the inhibition was competitive with dTTP (Fig. 5 inset). The apparent K_m value of DNA polymerase β for dTTP was 80 μM; the K_i for d_2TTP was approximately 0.15 μM.

Effects of concentrations of DNA and enzyme on inhibition by d_2TTP. The inhibition by d_2TTP of the DNA polymerase β activity was examined at various concentrations of the activated DNA template. At low substrate concentrations (5 μM [^{3}H]dTTP for DNA polymerase α and 10 μM [^{3}H]dTTP for DNA polymerase β) the inhibition by d_2TTP decreased with increasing concentrations of the activated DNA template; however, this relationship disappeared at higher substrate concentrations. Increasing enzyme concentration in the reaction mixture did not alter significantly the extent of d_2TTP inhibition.

Effects of Mg^{2+} and Mn^{2+} concentrations on inhibition by d_2TTP. With $(dT)_{\sim 15}\cdot(dA)_n$ primer template, the DNA polymerase α and β activities can be assayed using either Mg^{2+} or Mn^{2+}; however, at optimum concentrations of the metal ion, the enzyme activity with Mn^{2+} was only 45 per cent of that with Mg^{2+} (100 per cent activity corresponded to 26.6 and 22.2 pmoles of [^{3}H]dTMP incorporation by DNA polymerase α and β, respectively). The d_2TTP was more inhibitory when Mn^{2+} was used in the assay with $(dT)_{\sim 15}\cdot(dA)_n$ primer template. For example, 2.5 μM d_2TTP inhibited 72 per cent of the DNA polymerase β activity with Mn^{2+}, while the same amount of d_2TTP inhibited only 51.2 per cent of the activity with Mg^{2+}. Under similar conditions, the DNA polymerase α activity was inhibited only by 9.3 per cent with Mg^{2+} and 44.4 per cent with Mn^{2+}. Increasing the concentrations of either metal ion, however, did not bring about any change in the extent of inhibition by d_2TTP.

Preincubation. To determine whether preincubation of the inhibitor with either the enzyme or the template was necessary, it was mixed with either the enzyme or the activated DNA template and maintained at 37° for varying times; other ingredients were added following the preincubation period and the polymerase reaction was performed as usual. We found that preincubation was not required for the d_2TTP inhibition.

DISCUSSION

Studies with purified *E. coli* DNA polymerase have shown that d_2TTP binds to the triphosphate site and is a substrate for addition to a DNA chain. The rate of attachment was reported to be 0.1 per cent of the rate of attachment of natural nucleotide residues [5]. The terminal d_2TTP residue with no hydroxyl group is an effective covalent blocking group, stabilizing the primer end against hydrolysis, pyrophosphorolysis or polymerization. Although the details of the d_2TTP inhibition of bacterial DNA polymerases are known, very little is known about its inhibition of mammalian DNA polymerases.

The exact biological roles of DNA polymerase α and DNA polymerase β of mammalian cells are not understood. DNA polymerase α, by inference, is considered to be involved in DNA replication; DNA polymerase β is implicated in DNA repair synthesis [1–3]. Furthermore, while studying the processive mechanism of *E. coli* DNA polymerase I, Bambara *et al.* [15] suggested that DNA polymerase β from human KB cells is non-processive, i.e. the enzyme–DNA complex dissociates after addition of each nucleotide. Therefore, it was of interest to us to compare the effect of a chain terminator such as

d2TTP on the activities of DNA polymerase α and DNA polymerase β. Many inhibitors of DNA synthesis inhibit DNA polymerase α more than DNA polymerase β [4,16]. However, we noticed that the d2TTP was more inhibitory to DNA polymerase β than to DNA polymerase α. This is consistent with observations by Edenberg *et al.* [10], who reported during the course of our investigation that SV 40 DNA replication involving DNA polymerase α is resistant to d2TTP and that the DNA polymerase β of the CV-1 cells was more sensitive than the DNA polymerase α to d2TTP inhibition. Waqar *et al.* [11] also reported similar differences in sensitivity to d2TTP inhibition of DNA polymerase α and DNA polymerase β isolated from HeLa cells. The reason for the difference in sensitivity to d2TTP is not known. The resistance of DNA polymerase α to d2TTP inhibition was not due to exonuclease activity associated with DNA polymerase α; neither DNA polymerase α nor DNA polymerase β had any detectable exonuclease activity; furthermore, in mixing experiments, i.e. when d2TTp inhibition was tested with both DNA polymerase α and DNA polymerase β in the reaction mixture, there was no change in the extent of d2TTP inhibition. It may be possible to use this difference in their sensitivity to d2TTP inhibition to study the difference in the mechanism of DNA replication catalyzed by these polymerases [1].

It has been reported that the nuceloside, dideoxythymidine, can enter mouse cells, be phosphorylated, and be incorporated into replicating DNA [17]; this suggests that the pyrimidine kinase of mammalian cells may be able to phosphorylate the nucleoside analog. It may be of interest, therefore, to determine whether there is any difference in the effect of d2TTP on the growth of certain cell lines derived from patients with acute leukemia and lymphoma, and then to correlate the effects of the nucleoside analogs on cell growth and activity of the different polymerase species isolated from them.

Acknowledgements—I thank Dr. J. R. Bertino for his helpful criticisms. The technical assistance of Mr. S. A. Hasan and Mr. M. Borst is gratefully acknowledged.

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