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Stereospecificity of human DNA polymerases α, β, γ, δ and ε, HIV-reverse transcriptase, HSV-1 DNA polymerase, calf thymus terminal transferase and *Escherichia coli* DNA polymerase I in recognizing D- and L-thymidine 5′-triphosphate as substrate

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ABSTRACT

L-β-Deoxythymidine (L-dT), the optical enantiomer of D-β-deoxythymidine (D-dT), and L-enantiomers of nucleoside analogs, such as 5-iodo-2′-deoxy-L-uridine (L-IdU) and E-5-(2-bromovinyl)-2′-deoxy-L-uridine (L-BVdU), are not recognized *in vitro* by human cytosolic thymidine kinase (TK), but are phosphorylated by herpes simplex virus type 1 (HSV-1) TK and inhibit HSV-1 proliferation in infected cells. Here we report that: (i) L-dT is selectively phosphorylated *in vivo* to L-dTMP by HSV-1 TK and L-dTMP is further phosphorylated to the di- and triphosphate forms by non-stereospecific cellular kinases; (ii) L-dTTP not only inhibits HSV-1 DNA polymerase *in vitro*, but also human DNA polymerases α, γ, δ and ε, human immunodeficiency virus reverse transcriptase (HIV-1 RT), *Escherichia coli* DNA polymerase I and calf thymus terminal transferase, although DNA polymerase β was resistant; (iii) whereas DNA polymerases β, γ, δ and ε are unable to utilize L-dTTP as a substrate, the other DNA polymerases clearly incorporate at least one L-dTMP residue, with DNA polymerase α and HIV-1 RT able to further elongate the DNA chain by catalyzing the formation of the phosphodiester bond between the incorporated L-dTMP and an incoming L-dTTP; (iv) incorporated L-nucleotides at the 3′-OH terminus make DNA more resistant to 3′→5′ exonucleases. In conclusion, our results suggest a possible mechanism for the inhibition of viral proliferation by L-nucleosides.

INTRODUCTION

Despite the stereospecificity of enzymes, we have recently reported that L-β-deoxythymidine (L-dT), the optical enantiomer of the naturally occurring nucleoside D-β-deoxythymidine (D-dT), and L-enantiomers of nucleoside analogs, such as 5-iodo-2′-deoxy-L-uridine (L-IdU) and E-5-(2-bromovinyl)-2′-deoxy-L-uridine (L-BVdU), are selectively phosphorylated *in vitro* by the herpes simplex virus type 1 (HSV-1) thymidine kinase (TK) and inhibit HSV-1 proliferation in infected cells, but are not recognized by human cytosolic TK (1,2). The molecular basis for the selective inhibition of HSV-1 replication by L-dT and L-nucleoside analogs has not yet been elucidated. It is not known whether L-dTMP, which *in vitro* is not transformed to L-dTDP by viral TK (3), is further phosphorylated in HSV-1-infected cells by non-stereospecific cellular kinases to the di- and triphosphate forms and whether this latter may block viral proliferation by inhibiting the viral DNA polymerase or even by being incorporated into the viral genome, or both.

In the present work we addressed these questions by: (i) verifying whether L-dTMP could be further phosphorylated *in vivo* to di- and triphosphate forms; (ii) studying the kinetics of interaction of L-dTTP with the HSV-1 DNA polymerase, human DNA polymerases α, β, γ, δ and ε and, for comparison, with other DNA polymerases, such as the human immunodeficiency virus reverse transcriptase (HIV-1 RT), *Escherichia coli* DNA polymerase I and calf thymus terminal transferase; (ii) studying by primer extension and polyacrylamide DNA sequencing gel assays, the capability of the DNA polymerases to incorporate one L-dTMP residue and to further elongate the DNA chain by catalyzing formation of a phosphodiester bond between the incorporated L-dTMP and an incoming L-dTTP; (iv) verifying whether the incorporation of one or more L-nucleotides at the 3′-OH terminus makes DNA more resistant to 3′→5′ exonucleases.

Our studies demonstrate that the lack of stereodifferentiation is a property of several DNA polymerases and suggest a possible mechanism for the inhibition of viral proliferation by L-nucleosides.

MATERIALS AND METHODS

Chemicals and enzymes

[^{3}H]D-dT (30 Ci/mmol) was from Amersham. [^{3}H]L-dT (6 Ci/mmol) was from Moravek Biochemicals. Deoxyribonucleoside

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and ribonucleoside triphosphates were from Amersham [^{3}H-labeled] or from Boehringer (unlabeled). L-dTTP was synthesized from L-dThy (1) according to the procedure described by Ludwig and Ecstein (4). Thin-layer chromatography (TLC) polyethyleneimine (PEI)–cellulose F plastic sheets (layer thickness 0.1 mm) were from Merck. Dithiothreitol (DTT) was from Sigma. Bovine serum albumin (BSA) was from Calbiochem. All other chemicals and reagents (analytical grade) were purchased from local suppliers. Human DNA polymerases α, β and ε were purified from HeLa cells through three chromatographic steps, namely phosphocellulose, hydroxyapatite and heparin–Sepharose, as outlined in Focher *et al.* (5–7). Human DNA polymerase δ was purified from HeLa cells according to Weiser *et al.* (8). DNA polymerase γ was purified from HeLa cells according to Hübscher *et al.* (9). HSV-1 DNA polymerase was purified from HSV-1-infected HeLa cells as previously described (10). HIV-1 RT was kindly supplied by M. Hottiger and U. Hübscher (University of Zurich-Irchel, Switzerland). Calf thymus terminal transferase, *E.coli* DNA polymerase I (Klenow fragment), *E.coli* 3′→5′ exonuclease III and T4 polynucleotide kinase were purchased from Boehringer. Oligo(dT)$_{20}$ was synthesized using an Applied Biosystem 391 DNA Synthesizer.

Quantitation of L-dTMP, L-dTDP and L-dTTP in culturated human cells

HeLa and HeLa TK$^-$/HSV-1 TK$^+$ cells growing in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum at 37°C were collected, washed in phosphate-buffered saline (PBS), resuspended in DMEM without fetal calf serum at a concentration of 2 × 10^6 cells/ml and incubated at 37°C for 20 min before addition of 16 μM [^{3}H]D-dT (850 c.p.m./pmol) or 16 μM [^{3}H] L-dT (660 c.p.m./pmol) and 20 μg/ml aphidicolin (to inhibit incorporation of labeled dT into DNA by replicative DNA polymerases). After further incubation for the period of time indicated in Figure 1, cells (2 × 10^6) were harvested from each tube, centrifuged and washed in cold PBS in order to remove excess thymidine. Cellular pellets were then extracted in 100 μl 0.5 M percloric acid and centrifuged for 10 min at 10 000 r.p.m. in an Eppendorf microfuge. The acid-soluble material was neutralized, made 0.8 M with respect to ammonium acetate, pH 8, and centrifuged at 10 000 r.p.m. for 10 min. The supernatants were concentrated to 5–10 μl by lyophilization and 2 μl of each solution was run on PEI–cellulose TLC in 100% methanol (first run) and then in 44% methanol, 1.1 M LiCl, 0.37 M sodium formate, pH 3.4 (second run). In a parallel lane 1 μl of a mixture of dT, dTMP, dTDP and dTTP (each 20 mM) was used as an optical marker. The TLC sheet was then dried and observed under UV light. Each lane was cut into seven pieces according to the position of the markers and radioactivity was counted in a scintillation counter in 5 ml Betamax scintillation fluid (ICN).

Enzyme assays for kinetic analysis

DNA polymerases α, δ and ε were assayed at 37°C in 25 μl 50 mM Bis–Tris, pH 6.6, 10 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 250 μg/ml BSA, 0.3 μM [3′-OH ends] poly(dA)$_n$/oligo(dT)$_{20}$ (base ratio 30:1) and varying concentrations of [^{3}H]dTTP (500 c.p.m./pmol). The reaction mixture for DNA polymerase δ also included 100 ng PCNA.

DNA polymerase β was assayed at 37°C in 25 μl 50 mM Tris–HCl, pH 8.6, 10 mM $MgCl_2$, 100 mM KCl, 1 mM DTT, 0.25 mg/ml BSA, 0.3 μM [3′-OH ends] poly(dA)$_n$/oligo(dT)$_{20}$ (base ratio 30:1) and varying concentrations of [^{3}H]dTTP (500 c.p.m./pmol).

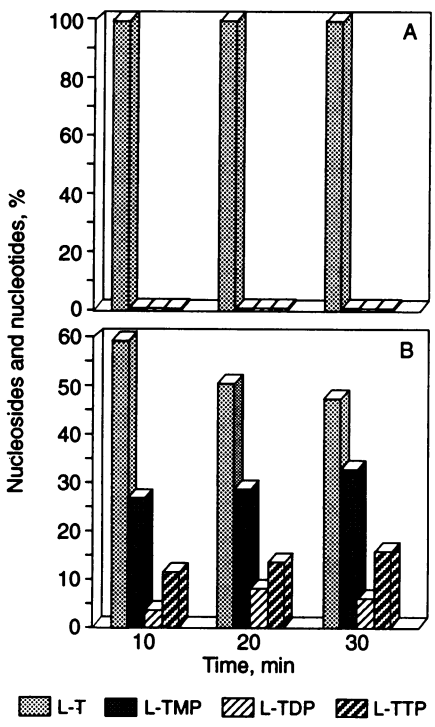

Figure 1. Percentage of ^{3}H-labelled L-dT, L-dTMP, L-dTDP and L-dTTP extracted from HeLa (**A**) and HeLa TK$^-$/HSV-1 TK$^+$ (**B**) cells after 10, 20 and 40 min incubation at 37°C in the presence of [^{3}H]L-dT. Experimental details as in Material and Methods.

DNA polymerase γ was assayed at 37°C in 25 μl 50 mM Tris–HCl, pH 8.6, 50 mM KPO_4, pH 8.6, 100 mM KCl, 0.5 mM $MnCl_2$, 1 mM DTT, 0.25 mg/ml BSA, 0.3 μM [3′-OH ends] poly(rA)$_n$/oligo(dT)$_{20}$ (base ratio 30:1) and varying concentrations of [^{3}H]dTTP (500 c.p.m./pmol).

HSV-1 DNA polymerase was assayed at 37°C in 25 μl 50 mM Tris–HCl, pH 8, 250 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mg/ml BSA, 0.3 μM [3′-OH ends] poly(dA)$_n$/oligo(dT)$_{20}$ (base ratio 30:1) and varying concentrations of [^{3}H]dTTP (500 c.p.m./pmol).

HIV-1 RT was assayed at 37°C in 25 μl 50 mM Tris–HCl, pH 8, 100 mM KCl, 5 mM $MgCl_2$, 0.05% NP-40, 0.3 μM [3′-OH ends] poly(rA)$_n$- or poly(dA)$_n$/oligo(dT)$_{20}$ (base ratio 30:1) and varying concentrations of [^{3}H]dTTP (500 c.p.m./pmol).

Calf thymus terminal transferase was assayed at 37°C in 25 μl 200 mM potassium cacodylate, 25 mM Tris–HCl, pH 6.6, 0.25 mg/ml BSA, 0.15 μM oligo(dT)$_{20}$ and varying concentrations of [^{3}H]dTTP (500 c.p.m./pmol).

Escherichia coli DNA polymerase I (Klenow fragment; Boheringer) was assayed at 37°C in 25 μl of the reaction mixture suggested by Boheringer in the presence of 0.3 μM [3′-OH ends] poly(dA)$_n$/oligo(dT)$_{20}$ (base ratio 30:1) and varying concentrations of [^{3}H]dTTP (500 c.p.m./pmol).

Escherichia coli 3′→5′ exonuclease III (Boheringer) was assayed at 37°C in 25 μl 66 mM Tris–HCl, pH 7.6, 1 mM $MgCl_2$ and 1 mM DTT.

The reactions were terminated by spotting 20 μl of the incubation mixture on a 25 mm GF/C filter (Whatman). The

filters were washed three times in 5% (v/v) TCA for 5–10 min and twice in ethanol in order to remove the acid-soluble material. The radioactivity incorporated into DNA was estimated in 1 ml Betamax scintillation fluid (ICN) in a scintillation counter.

Primer extension and polyacrylamide gel assay

Oligo(dT)$_{20}$ primer was labeled by incubating equal quantities of the primer (as 5′-OH) and [γ-^{32}P]ATP (3000 Ci/mmol) with T4 polynucleotide kinase (Boheringer) under the conditions suggested by Boheringer. The primer was annealed to poly(dA) or poly(A) at a base ratio of 1:30 by heating at 90°C for 5 min, followed by gradual cooling to room temperature. For each DNA polymerase the DNA elongation reaction was performed in 10 µl of the reaction mixture described above, using the concentrations of D- or L-dTTP indicated in the figures. After incubation at 37°C reactions were terminated by adding an equal volume of Stop Solution (United States Biochemicals). Samples were denatured by boiling for 3 min and loaded on 20% polyacrylamide–8 M urea DNA sequencing gels. Electrophoresis was carried out at 40 W for 3–4 h. The radioactivity was analyzed by autoradiography using Hyperfilm MP (Amersham).

RESULTS

L-Thymidine is converted to the triphosphate form in HeLa TK$^-$/HSV-1 TK$^+$ cells

In a previous work we have demonstrated that L-dT is selectively phosphorylated to L-dTMP by HSV-1 TK and inhibits viral growth (1). To verify whether L-dTMP could be further metabolized to the di- and triphosphate forms in an intact cellular system we followed the fate of L-dT and D-dT in both wild-type HeLa cells and in HeLa TK$^-$ cells transformed with the HSV-1 TK gene (HeLa TK$^-$/HSV-1 TK$^+$).

For this purpose [^{3}H]L-dT or [^{3}H]D-dT was added to HeLa and to HeLa TK$^-$/HSV-1 TK$^+$ cells in the presence of 20 µg/ml aphidicolin, which inhibits the incorporation of dT into DNA by DNA polymerases. The amounts of the mono-, di- and triphosphate forms were determined by the method described in Materials and Methods. In both cell lines we observed that D-dT is rapidly transformed into D-dTTP (data not shown). On the other hand, L-dT was phosphorylated to the mono-, di and triphosphate forms only in the cell line carrying the HSV-1 TK gene (Fig. 1). These results prompted us to verify whether L-dTTP could interfere with eukaryotic, prokaryotic and viral DNA polymerases *in vitro*.

Kinetic analysis of the inhibitory activity of L-dTTP on human DNA polymerases α, β and γ, HIV-1 RT, HSV-1 DNA polymerase, calf thymus terminal transferase and *E.coli* DNA polymerase I

The effect of L-dTTP on various DNA polymerases was tested with poly(dA)$_n$/oligo(dT)$_{20}$ or poly(rA)$_n$/oligo(dT)$_{20}$ under conditions optimal for each enzyme (see Materials and Methods). The template primer utilized, the K_m for D-dTTP and inhibition constants (K_i) for L-dTTP, as well as the type of inhibition, for each DNA polymerase are summarized in Table 1.

Table 1. K_m for substrates and template primers (3′-OH) and K_i of L-dTTP on DNA polymerases α, γ, δ and ε, HSV-1 DNA polymerase, HIV-1 RT, *E.coli* DNA polymerase I and terminal transferase on homopolymeric DNA templates

DNA polymerase (template primer)	Variable substrate	K_m[a] (µM)	K_i[a] (µM)	Inhibition
DNA polymerase α				
poly(dA)$_n$/oligo(dT)$_{20}$	D-dTTP	10.9	286	Competitive
DNA polymerase γ				
poly(rA)$_n$/oligo(dT)$_{20}$	D-dTTP	5.2	8	Competitive
DNA polymerase δ				
poly(dA)$_n$/oligo(dT)$_{20}$	D-dTTP	10.4	200	Non-competitive
	3′-OH	0.036	72	Non-competitive
	PCNA	2.2 (µg/ml)	50	Mixed type
DNA polymerase ε				
poly(dA)$_n$/oligo(dT)$_{20}$	D-dTTP	5.8	257	Non-competitive
	3′-OH	0.12	14	Competitive
HSV-1 DNA polymerase				
poly(dA)$_n$/oligo(dT)$_{20}$	D-dTTP	2.8	33	Competitive
HIV-1 RT				
poly(rA)$_n$/oligo(dT)$_{20}$	D-dTTP	8.3	105	Competitive
poly (dA)$_n$/oligo (dT)$_{20}$	D-dTTP	15	203	Competitive
Terminal transferase				
oligo(dT)$_{20}$	D-dTTP	100	60	Competitive
E.coli DNA polymerase I				
poly(dA)$_n$/oligo(dT)$_{20}$	D-dTTP	5	70	Competitive

[a]Regression lines were obtained using the HyperCard program Enzyme kinetics (D.G.Gilbert, Indiana University). Competitive and non-competitive K_i values were obtained from $K_i = I/[(K_m'/K_m) - 1]$ (where K_m' is the apparent K_m of the variable substrate in the presence of inhibitor at concentration I) and from $K_i = I/[(V_m/V_m') - 1]$ (where V_m' is the apparent V_m in the presence of inhibitor at concentration I) respectively. Each value is the average of three experiments.

Human DNA polymerases α, γ, HIV RT, HSV-1 DNA polymerase, *E.coli* DNA polymerase I and calf thymus terminal transferase are inhibited competitively when D-dTTP is the variable substrate. Among them, DNA polymerase γ is the most sensitive enzyme, with a K_i for L-dTTP (8 μM) very close to the K_m for D-dTTP (5.2 μM), suggesting that the binding of a dNTP to the active site of this enzyme is not a stereospecific reaction, being equally effective with the unnatural L-dTTP. DNA polymerase α and HSV-1 DNA polymerase were competitively inhibited by L-dTTP, with K_i values of 286 and 33 μM respectively. The K_i values of HIV RT for L-dTTP were ~100 and 200 μM on RNA-dependent and DNA-dependent DNA synthesis respectively. Terminal transferase and *E.coli* DNA polymerase I were also inhibited by L-dTTP in a competitive way, with K_i of 60 and 70 μM respectively.

DNA polymerase β, assayed on poly(dA)$_n$/oligo(dT)$_{20}$, was resistant to L-dTTP up to 1 mM (highest tested concentration).

Kinetic analysis of the inhibitory activity of L-dTTP on human DNA polymerases δ and ε

The kinetics of the interaction of L-dTTP with DNA polymerases δ and ε appeared more complex. In fact, L-dTTP inhibited DNA polymerase δ activity in a non-competitive way when D-dTTP (data not shown) or DNA template were the variable substrates (Fig. 2A and Table 1), with K_i values of 200 and 72 μM respectively. However, in the presence of varying concentrations of proliferating cell nuclear antigen (PCNA), the auxiliary protein required by DNA polymerase δ for its activity (11), L-dTTP showed a mixed type inhibition, with a K_i of 50 μM (Fig. 2B and Table 1).

DNA polymerase ε is also inhibited by L-dTTP in a non-competitive way when D-dTTP was the variable substrate (Fig. 3A and Table I), but when assays were carried out in the presence of varying concentrations of template primer, L-dTTP acted as a competitive inhibitor (Fig. 3B and Table 1).

A possible mechanism for the inhibition of DNA polymerases δ and ε by L-dTTP, consistent with these results, is presented in the Discussion.

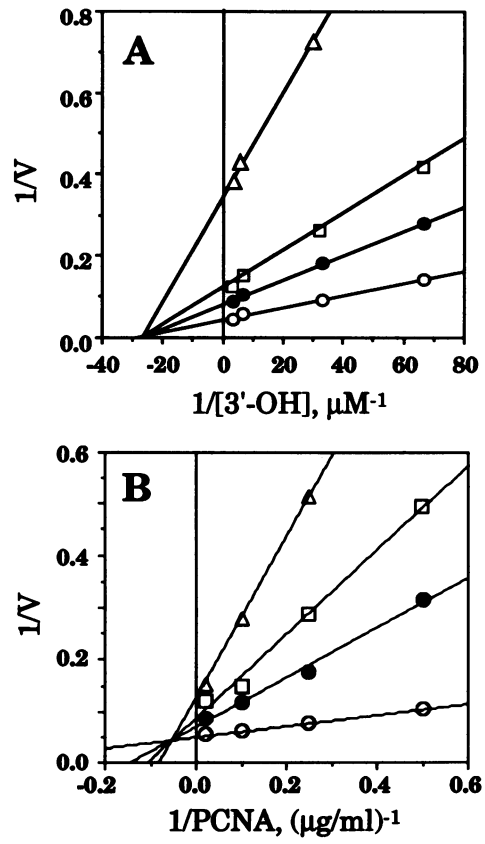

Figure 2. Lineweaver–Burk plot of the effect of L-dTTP on human DNA polymerase δ activity in the presence of different concentrations of template primer (3′-OH end) (**A**) or PCNA (**B**). In the experiments reported in (A) PCNA was present in the assay at the fixed concentration of 0.1 μg/ml and L-dTTP concentrations were: (○) 0, (●) 50, (□) 150 and (△) 500 μM. In the experiments reported in (B) DNA template (3′-OH end) was present in the assay at the fixed concentration of 0.3 μM and L-dTTP concentrations were: (○) 0, (●) 50, (□) 150 and (△) 300 μM.

Studies on primer extension by human DNA polymerases α, β, γ, δ, ε, HSV-1 DNA polymerase, HIV-1 RT, terminal transferase and *E.coli* DNA polymerase I (Klenow fragment) in the presence of L-dTTP

To verify whether L-dTTP is a non-substrate or a substrate inhibitor of human DNA polymerases α, β, γ, δ and ε, HSV-1 DNA polymerase, HIV-1 RT, terminal transferase and *E.coli* DNA polymerase I, we have used the primer extension assay described in Materials and Methods. Whereas DNA polymerases β, γ, δ and ε are unable to incorporate L-dTTP (Fig. 4), one L-dTMP residue is clearly incorporated by the other DNA polymerases tested (Fig. 5). DNA polymerase α and *E.coli* DNA polymerase I (Klenow fragment) also incorporated at least a second L-dTMP residue. HIV-1 RT is the least stereospecific DNA polymerase, being able to incorporate 3–4 L-dTMP residues. The lack of incorporation of L-dTMP by DNA polymerases β, γ, δ and ε occurs even under conditions favoring non-processive DNA synthesis (12), which reduce the formation of long DNA fragments, but which should favor the frequent formation of the enzyme–template primer–L-dTTP complex. Even though our results indicate that these DNA polymerases do not have the intrinsic ability to incorporate L-dTMP, it cannot be ruled out that under conditions of higher catalytic efficiency, i.e. closer to the *in vivo* situation, one or a few L-dTMP residues could also be incorporated by these DNA polymerases.

However, the results are surprising only for DNA polymerase γ, because DNA polymerase β is resistant to L-dTTP and DNA polymerases δ and ε are inhibited in a non-competitive way when D-dTTP is the variable substrate (Table 1).

For permissive DNA polymerases the incorporation of one or more L-dTMP residues occurs at low concentrations of L-dTTP. The possibility that the observed incorporation of L-dTMP residues is due to contamination by the D-enantiomer is unlikely, because L-dTTP is obtained from L-thymidine by a process which does not involve the chiral center of the molecule. Thus its enantiomeric purity must be, at least, equal to that of the starting L-thymidine, estimated to be >99.7% by the extremely sensitive assay with purified HeLa TK (2). Furthermore, non-permissive DNA polymerases (Fig. 5) do not incorporate L-dTMP residues even at 1000 μM L-dTTP (the highest concentration used) and poly(dA)$_n$/[^{32}P]oligo(dT)$_{20}$ extended with L-dTMP residues appears more resistant to exonucleolytic degradation (see below).

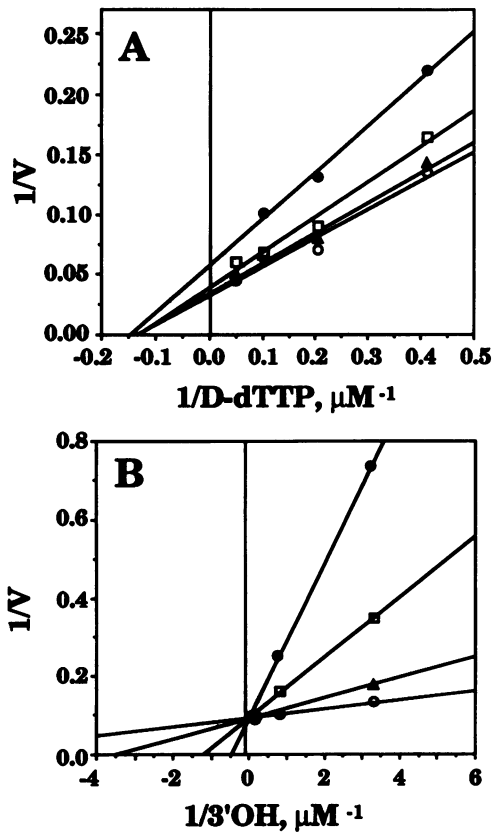

Figure 3. Lineweaver–Burk plot of the effect of L-dTTP on human DNA polymerase ε in the presence of different concentrations of D-dTTP (**A**) or template primer (**B**). In the experiments reported in (A) template primer was present in the assay at the fixed concentration of 0.3 μM (as 3′-OH ends); in the experiments reported in (B) D-dTTP was present in the assay at the fixed concentration of 10 μM. L-dTTP concentrations: (○) 0, (▲) 20, (□) 50 and (●) 150 μM.

It was recently shown that a synthetic polynucleotide with three terminal L-dCMP residues appears able to form a duplex with a complementary natural polynucleotide (13). Thus the observed incorporation of a single L-dTMP residue by DNA polymerases may still allow DNA duplex formation. Our data with HIV-1 RT and DNA polymerase α indicate that this L-terminal primer can also be extended by permissive DNA polymerases.

With HSV-1 DNA polymerase we could not verify whether L-dTTP is a substrate or a non-substrate inhibitor using poly(dA)$_n$/[^{32}P]oligo(dT)$_{20}$ as template primer, due to the rapid removal of ^{32}P from the 5′-end of the oligo(dT)$_{20}$ by the potent 5′→3′ exonuclease, which is an intrinsic component of the 136 kDa DNA polymerase polypeptide (14).

An L-dTMP residue at the 3′-primer terminus makes the primer more resistant to 3′→5′ exonuclease activity

In order to test the effect of an L-nucleotide residue at the 3′-primer terminus on 3′→5′ exonucleolytic activity oligo(dT)$_{17-20}$ was ^{32}P-labeled at the 5′-terminus and, after annealing with poly(dA), was elongated by DNA polymerase α in the presence of 0.5 μM D-dTTP or 500 μM L-dTTP as described in Material and Methods. After 30 min incubation at 37°C, when ~50% of the oligo(dT)$_{17-20}$ has been extended with 1–4 D- or L-dTMP residues (Fig. 6), reaction mixtures were heated at 65°C for 5 min, to inactivate DNA polymerase α, and divided into aliquots which were further incubated at 37°C for 30 min with different amounts of *E.coli* 3′→5′ exonuclease III, indicated in Figure 6. Samples were then denatured by boiling for 3 min and loaded on 20% polyacrylamide–8 M urea DNA sequencing gels. As shown in Figure 6, at high concentration of exonuclease III poly(dA)$_n$/[^{32}P]oligo(dT)$_{17-20}$ extended with either D- or L-dTMP residues is fully degraded. At lower concentrations the rate of hydrolysis of poly(dA)$_n$/[^{32}P]oligo(dT)$_{17-20}$ by *E.coli* 3′→5′ exonuclease III is significantly lower when L-dTMP residues are incorporated at the 3′-primer terminus by DNA polymerase α, as suggested by the higher amounts of resulting [^{32}P]oligo(dT)$_{13-20}$ at various exonuclease III concentrations. Quantitative results will be possible with a synthetic oligonucleotide chemically extended with two or three 3′-terminal L-dTMP residues. Our results, however, suggest that L-nucleotides at the 3′-terminus most probably increase the stability of [^{32}P]oligo(dT)$_{20}$ to 3′→5′ exonucleolytic degradation.

DISCUSSION

Previously we have found that L-dT, the optical enantiomer of the naturally occurring nucleoside D-dT, and L-enantiomers of nucleoside analogs, such as L-IdU and L-BVdU, are selectively phosphorylated by purified HSV-1 TK and inhibit HSV-1 proliferation in infected cells (1,2). In the present study we demonstrate that L-thymidine is selectively phosphorylated to L-dTMP only in HeLa TK$^-$/HSV-1 TK$^+$ cells in which thymidine phosphorylation depends on viral TK and that L-dTMP is further metabolized to L-dTDP and L-dTTP. Our data indicate that in intact cells HSV-1 TK is also clearly responsible for phosphorylation of L-dT to L-dTMP and that cellular nucleotide kinases are not or poorly stereospecific enzymes. The formation of L-dTTP in HSV-1-infected cells suggests that one mechanism for the inhibition of HSV-1 replication by L-dT and L-nucleoside analogs might be interaction of L-dTTP with viral DNA polymerase.

It is already known that the binding of 2′-deoxynucleoside 5′-triphosphates by DNA polymerases is determined by the enzyme, as well as being dictated by the DNA sequence of the template. Template primer binding by DNA polymerases in fact precedes binding of the template-oriented 2′-deoxynucleoside-5′-triphosphate, before DNA polymerase catalyzes stereospecific nucleophilic attack of the 3′-OH primer terminus on the α-phosphate of the incoming dNTP. For binding and subsequent utilization of dNTPs by DNA polymerases significant changes in the base, sugar and, to a much lesser extent, in the triphosphate moiety can be tolerated if they do not alter their base pairing potentiality. Analogs of 2′-deoxyribonucleotides in which the altered base can form unorthodox base pairs can still bind and serve as substrates for DNA polymerases (15). Thus it was of interest to study how the inverted configuration of the deoxyribosyl moiety would affect interaction with DNA polymerases. Indeed, our study demonstrates that L-dTTP interacts with HSV-1 DNA polymerase as well as with human DNA polys α, γ, δ and ε and other DNA polymerases, such as HIV-1 RT, *E.coli* DNA polymerase I and terminal transferase.

In particular, our data indicate that L-dTTP competitively inhibits, with respect to D-dTTP, HSV-1 DNA polymerase, human DNA polymerases α and γ, HIV-1 RT, terminal transferase

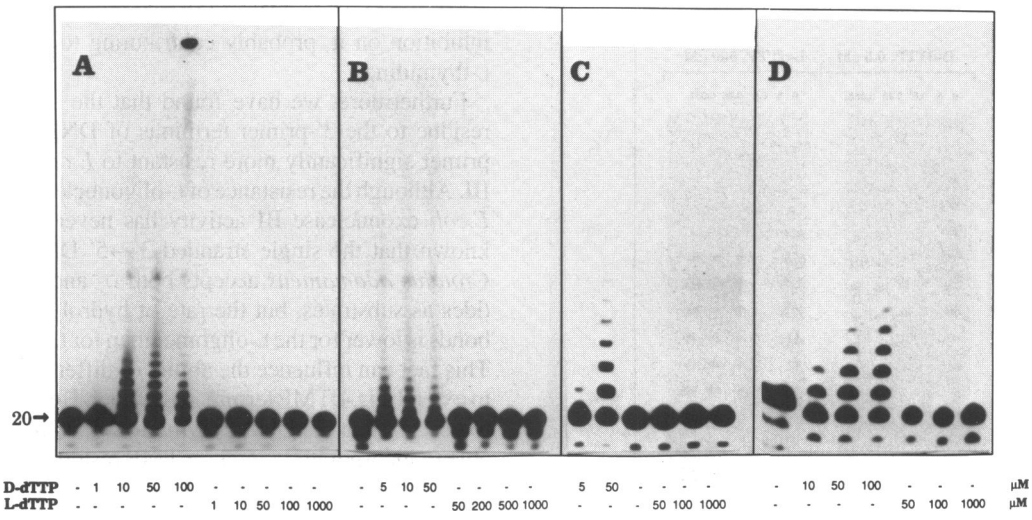

Figure 4. Primer extension by DNA polymerase β (**A**), DNA polymerase γ (**B**), DNA polymerase δ (**C**) and DNA polymerase ε (**D**) using different concentrations of D-dTTP or L-dTTP on poly(dA)$_n$/oligo(dT)$_{20}$ (A, C and D) or poly(rA)$_n$/oligo(dT)$_{20}$ (B). DNA polymerase assays, the primer extension assay and the polyacrylamide DNA sequencing gel procedure are described in Materials and Methods. Arrow indicates the position of the 20 nt unextended primer.

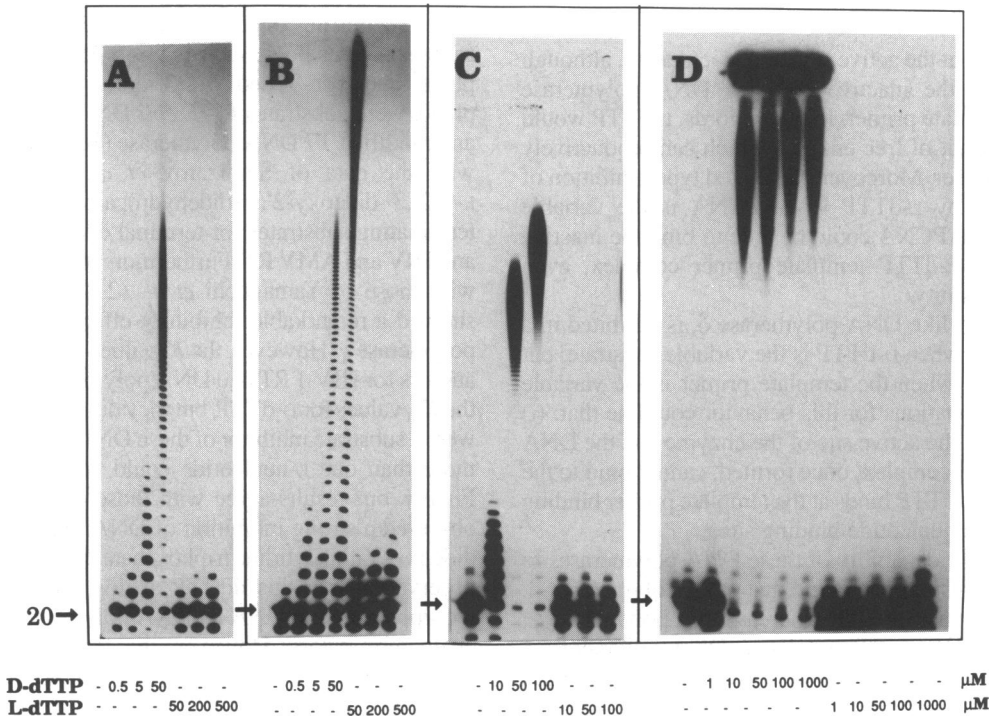

Figure 5. Primer extension by DNA polymerase α (**A**), HIV-1 RT (**B**), calf thymus terminal transferase (**C**) and *E.coli* DNA polymerase I, Klenow fragment (**D**) using different concentrations of D-dTTP or L-dTTP on poly(dA)$_n$/oligo(dT)$_{20}$ (A and D) poly(rA)$_n$/oligo(dT)$_{20}$ (B) or oligo(dT)$_{20}$ (C). DNA polymerase assays, the primer extension assay and the polyacrylamide DNA sequencing gel procedure are described in Materials and Methods. Arrow indicates the position of the 20 nt long unextended primer.

and *E.coli* DNA polymerase I. The kinetics of interaction of L-dTTP with DNA polymerases δ and ε appeared more complex. DNA polymerase δ was inhibited in a non-competitive way when D-dTTP or DNA were the variable substrates and in a mixed type manner when PCNA was the variable substrate. Primer recognition by DNA polymerase δ follows an ordered sequential mechanism, in which the template primer binds first and then the enzyme–template primer complex is stabilized by PCNA, followed by dNTP binding and chemical bond formation (16). Therefore, the non-competitive inhibition observed when the template primer is the variable substrate suggests that L-dTTP does not follow the above sequential order, but can bind to the free

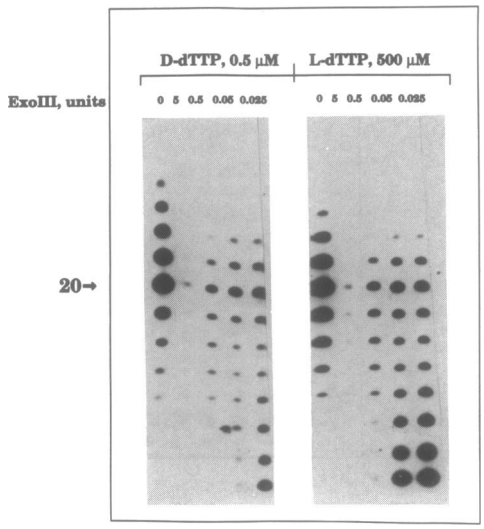

Figure 6. Effect of L-dTMP at the 3′-terminus of [^{32}P]oligo(dT)$_{20}$ on *E.coli* 3′→5′ exonuclease III activity. Experimental details are reported in Materials and Methods and in the text. Electrophoresis was carried out at 40 W for 3–4 h. Arrow indicates the position of the 20 nt unextended primer.

enzyme, probably not at the active site, and inactivate it, although allowing binding of the inactive complex DNA polymerase δ–L-dTTP to the template primer. In other words, L-dTTP would only reduce the amount of free enzyme which can productively bind the template primer. Moreover, the mixed type inhibition of DNA polymerase δ by L-dTTP when PCNA is the variable substrate suggests that PCNA could be able to bind the inactive DNA polymerase δ–L-dTTP–template primer complex, even though with lower affinity.

DNA polymerase ε, like DNA polymerase δ, is inhibited in a non-competitive way when D-dTTP is the variable substrate, but in a competitive way when the template primer is the variable substrate. Some explanations for this behavior could be that: (i) L-dTTP does not bind the active site of the enzyme and the DNA polymerase ε–L-dTTP complex, once formed, cannot bind to the template primer; (ii) L-dTTP binds at the template primer binding site, rather than at the nucleotide binding site.

When we studied the capability of these DNA polymerases to utilize L-dTTP as a substrate we found that DNA polymerases β, γ, δ and ε are unable to use L-dTTP, whereas DNA polymerase α, terminal transferase, HIV-1 RT and *E.coli* DNA polymerase I clearly incorporate one L-dTMP residue, with DNA polymerase α and HIV-1 RT being capable of additional elongation of the DNA chain by catalyzing formation of a phosphodiester bond between the incorporated L-dTMP and an incoming L-dTTP. These results are not unexpected, because DNA polymerase β is resistant to L-dTTP and DNA polymerases δ and ε are inhibited in a non-competitive way when D-dTTP is the variable substrate. Thus at the incorporation level the overall stereospecificity of DNA polymerases depends both upon the ability to bind L-dTTP and the ability to catalyze the formation of the phosphodiester bond.

The potent 5′→3′ exonuclease, an intrinsic component of the 136 kDa DNA polymerase polypeptide (14), did not allow verification of whether L-dTTP is or is not a substrate for HSV-1 DNA polymerase. However, even though L-dTTP may not be a substrate for this DNA polymerase, it exerts a very strong inhibition on it, probably contributing to the antiviral effect of L-thymidine.

Furthermore, we have found that the addition of a L-dTMP residue to the 3′-primer terminus of DNA makes the template primer significantly more resistant to *E.coli* 3′→5′ exonuclease III. Although the resistance of L-oligonucleotides to hydrolysis by *E.coli* exonuclease III activity has never been published, it is known that the single stranded 3′→5′ DNA exonuclease from *Crotalus adamanteus* accepts both D- and L-oligodeoxynucleotides as substrates, but the rate of hydrolysis of phosphodiester bonds is lower for the L-oligomer than for the D-oligomer (13,17). This fact can influence the ability of different DNA polymerases to extend an L-dTMP terminated primer. However, it is interesting to observe that the DNA polymerases that have been found to be able to incorporate several L-dTMP residues (i.e. DNA polymerase α and HIV-1 RT) lack a 3′→5′ exonuclease activity.

The results reported here seem surprising, because when the nucleobase portion of a L-β-nucleoside triphosphate is base paired to the DNA template strand in the DNA polymerase active site, its furanosyl portion is inverted relative to the orientation existing in the naturally occurring D-β-nucleoside triphosphate. However, they confirm our previous results (18) and are in agreement with the recent kinetic studies of Van Draanen *et al.* (19), suggesting that obligate chain-terminating nucleoside triphosphate analogs, such as β-L-3′-deoxythymidine 5′-triphosphate and β-L-3′-deoxy-2′,3′-didehydrothymidine 5′-triphosphate, were substrates for *E.coli* DNA polymerase I, HIV-1 RT and modified T7 DNA polymerase (Sequenase). They also agree with the data of Semizarov *et al.* (20), who showed that L-β-2′,3′-dideoxy-2′,3′-didehydrocarbocyclic dATP analogs are terminating substrates for terminal deoxynucleotidyl transferase and HIV and AMV RT. Furthermore, the results are in agreement with those of Yamaguchi *et al.* (21), who found that L-dTTP showed a remarkable inhibitory effect on HIV-1 RT and DNA polymerase γ. However, the K_i values for L-dTTP found by these authors for HIV-1 RT and DNA polymerase γ are even lower than the K_m values for D-dTTP, but they did not verify whether L-dTTP were a substrate inhibitor of these DNA polymerases and whether more than one L-nucleotide could be incorporated into DNA. Finally, our results agree with those of Chang *et al.* (22), who observed a strong inhibition of DNA polymerase γ by (–)-2′,3′-dideoxy-3′-thiacytidine triphosphate, but are in conflict with their observation that this ddCTP analog competes with dCTP for incorporation into DNA by DNA polymerases γ and β serving as chain terminators. However, the lack of hydroxyl groups in positions 2′ and 3′ of the nucleotide analog could reduce the steric impedence of the inverted sugar ring, allowing its accomodation in the active site of DNA polymerases (19). Alternatively, variations in the assay conditions may account for this discrepancy.

Our results suggest a possible mechanism for the inhibition of HSV-1 proliferation by L-dT: the previous observation that L-dT can be phosphorylated to L-dTMP only by the HSV-1 TK provides the basis for its selectivity (1), whereas the fact that L-dTMP can be phosphorylated *in vivo* to its triphosphate form by cellular kinases and that L-dTTP inhibits the viral DNA polymerase explain the final inhibitory effect on viral DNA synthesis.

From this study it appears that when D-dTTP is the variable substrate, HSV-1 DNA polymerase is nearly 10 times more

sensitive to L-dTTP than the cellular DNA polymerases α, δ and ε. Furthermore, the activity of cellular DNA polymerases and host DNA synthesis decline very rapidly early in HSV-1 infection (23). Therefore, in HSV-1-infected cells, where dTTP essentially derives from the activity of viral TK, treatment with L-dT will lead to an imbalance between the L-dTTP and D-dTTP pools, favoring binding of the former to the viral DNA polymerase, with consequent inhibition of viral DNA synthesis.

To summarize, inhibition of HIV-1 RT by L-nucleoside triphosphates and its ability to use them as substrates, here described, could represent the molecular basis of the reported anti-HIV activities of β-L-2′,3′-dideoxycytitdine, of its 5-fluoro derivative (24) and of other L-deoxycytosine analogs (22). From the lack of stereodifferentiation of human deoxycytidine kinase (umpublished results), these L-nucleoside analogs are probably phosphorylated to monophosphates by deoxycytidine kinase and then further phosphorylated by non-stereospecific cellular kinases to di- and triphosphates that interfere with HIV-1 RT.

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