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Interaction of β-L-2′,3′-dideoxy-2′,3′-didehydro-5-fluoro-CTP with Human Immunodeficiency Virus-1 Reverse Transcriptase and Human DNA Polymerases: Implications for Human Immunodeficiency Virus Drug Design

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ABSTRACT

The work reported in this article has evaluated the relative molecular activity of the 5′-triphosphate of a novel β-L-nucleoside with an unsaturated ribose residue, β-L-2′,3′-dideoxy-2′,3′-didehydro-5-fluorocytidine (β-L-Fd4CTP), with that of β-L-2′,3′-dideoxy-5-fluorocytidine (β-L-FddCTP) and 2′,3′-dideoxycytidine (ddCTP), on DNA strand elongation by human immunodeficiency virus-1 reverse transcriptase (HIV RT) and human DNA polymerases α (pol α), β (pol β), γ (pol γ), and ϵ (pol ϵ). The concentrations of β-L-Fd4CTP that inhibited the yield of products by 50% were 0.20 μM, 1.8 μM, and 4.0 μM for HIV RT, pol γ, and pol β, respectively. The β-L-Fd4CTP at a concentration as high as 40 μM had no inhibitory effect on pol ϵ, but could inhibit pol α by 10–20% at 20 μM. The K_m and relative V_{max} values of β-L-Fd4CTP, β-L-FddCTP, and ddCTP for incorporation into the standing start point of 5′-[^{32}P]-oligonucleotide primer annealed with M13mp19 phage DNA by HIV RT and human DNA polymerases were evaluated. The efficiency of incorporation (V_{max}/K_m) of β-L-Fd4CTP by HIV RT was about 4-fold and 12-fold higher than that of ddCTP and β-L-FddCTP, respectively. In contrast, the V_{max}/K_m ratio of β-L-Fd4CTP for pol γ was 7-fold lower than that of ddCTP, but 4-fold higher than that of β-L-FddCTP. Pol α could use β-L-Fd4CTP as a substrate, but only at a high concentration (>20 μM). Incorporation of β-L-Fd4CTP by pol ϵ could not be detected. A hypothesis about the preferable recognition of the 2′,3′-dideoxy-2′,3′-didehydro- structure of β-L-Fd4CTP to that of the 2′,3′-dideoxy-structure of β-L-FddCTP by HIV RT is discussed.

In the last several years, considerable effort has been devoted to the study of L-nucleoside analogs as anti-HIV and anti-HBV agents. Among them, 3TC and its 5-fluoro analog and β-L-2′,3′-dideoxycytidine and its 5-fluoro derivative, β-L-FddC, were demonstrated to have potent activity against both HIV and HBV (Belleau *et al.*, 1989; Doong *et al.*, 1991; Lin *et al.*, 1994a, 1994b). Recently, a novel L-nucleoside analog with an unsaturated ribose residue, β-L-2′,3′-dideoxy-2′,3′-didehydrocytidine and its 5-fluoro derivative, β-L-Fd4C, have been shown to possess even more potent anti-HIV and anti-HBV activity in cell culture than the compounds mentioned above (Lin *et al.*, 1996). It was suggested that the termination of viral DNA synthesis catalyzed by HIV RT or HBV DNA polymerase might be the major mechanism of the antiviral action. β-L-Fd4C was also reported to be more toxic than 3TC or β-L-FddC against the replication of various cell lines in culture (Lin *et al.*, 1996; Faray *et al.*, 1997). The underlying mechanism of the growth inhibitory activity by β-L-Fd4C may be attributed to its capacity to inhibit nuclear DNA synthesis. It is noteworthy that β-L-Fd4C is the first L-cytidine derivative with a 2′,3′-unsaturated sugar moiety discovered to have potent activity against HIV and HBV in cell culture. The X-ray analysis of β-D-2′,3′-dideoxy-2′,3′-didehydro-nucleosides and the interaction of their 5′-triphosphates with HIV RT and some human DNA polymerases have been studied (Dyatkina *et al.*, 1987; St. Clair *et al.*, 1987; Birnbaum *et al.*, 1989; Gurskaya *et al.*, 1991; Harte *et al.*, 1991; Van Roey and Chu, 1992). No information on X-ray analysis or on the molecular interaction of β-L-Fd4CTP with HIV RT and DNA polymerases has been reported previously.

In this work, we employed a DNA primer extension technique and sequencing gel analysis to evaluate the kinetic

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ABBREVIATIONS: HIV, human immunodeficiency virus type 1; HBV, human hepatitis B virus; pol, polymerase; RT, reverse transcriptase; dNTP, 2′-deoxynucleoside 5′-triphosphate; dCTP, 2′-deoxycytidine 5′-triphosphate; ddNTP, 2′,3′-dideoxynucleoside 5′-triposhate; ddC, 2′,3′-dideoxycytidine; ddCTP, 5′-triphosphate of ddC; FddC, 5′-fluoro-analog of ddC; FddCTP, 5′-triposphate of FddC; Fd4C, 2′,3′-dideoxy-2′,3′-didehydro-5-fluorocytidine; Fd4CTP, 5′-triphosphate of FddC; 3TC, β-L-2′,3′-dideoxy-3′-thiocytidine.

interactions of β-L-Fd4CTP with HIV-1 RT and human DNA polymerases α, β, γ, and ϵ. In this article, we also describe a novel procedure for the synthesis of β-L-Fd4CTP.

Materials and Methods

dNTPs and ddCTP were purchased from Boehringer Mannheim (Indianapolis, IN). β-L-FddC was a kind gift of the late Dr. T.S. Lin. β-L-FddCTP was synthesized from β-L-FddC in this laboratory. The synthesis of β-L-Fd4CTP is described below. The M13mp19(+) strand DNA was isolated as described previously (Sambrook *et al.*, 1989). The 22-base primer 5′-d(GTAAACGACGGCCGTGAATT-3′) was synthesized on an Applied Biosynthesis 380A DNA synthesizer at the Yale Oligonucleotide Synthesis Facility. The primer oligonucleotide was labeled at the 5′-position with T4 polynucleotide kinase using 3000 Ci/mM [γ-^{32}P]ATP (Amersham, Arlington Heights, IL) annealed to M13mp19 phage DNA as previously described (Sambrook *et al.*, 1989). The complex was purified on a Sephadex G-50 column, and used as a substrate for elongation reaction.

Human pol α, pol β, and pol γ were purified from chronic lymphocytic leukemia cells obtained from patients by leukophoresis. The procedure for purification and the characterization of human DNA polymerases were previously described (Kukhanova *et al.*, 1995). Pol ϵ from human placenta was a kind gift of Dr. D. Mozzherin (Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia). HIV RT was a kind gift of Dr. K. Anderson (Pharmacology Department, Yale University).

Synthesis of β-L-Fd4CTP. β-L-Fd4C used for the synthesis of β-L-Fd4CTP was prepared as described previously (Chen *et al.*, 1997). To the amidine-protected β-L-Fd4C (200 mg, 0.709 mmol) in 10 ml CH_2C1_2 and 3Å sieves was added 2-cyanoethyl diisopropyl chlorophosphoramidine (336 mg, 1.42 mmol) followed by diisopropylethylamine (336 mg, 2.8 mmol). The reaction mixture was stirred at room temperature for 2 hr Tributylammonium pyrophosphate (514 mg, 1.4 mmol) and 0.5 M tetrazole (2.9 ml, 1.4 mmol) were then added to the above mixture and stirred for 2 days. At this point *m*-chloroperbenzoic acid (612 mg, 3.5 mmol) in 5 ml CH_2C1_2 was added and the reaction was stirred for another 1 day. Finally, 2 M ammonia in methanol (0.37 ml, 2.36 mmol) was added to remove the protective groups. After removal of solvents, the crude product was dissolved in 0.2 ml deionized H_2O and applied to a 20 cm DEAE-cellulose column. The product was eluted with a linear gradient of 0 to 0.4 M triethylammonium bicarbonate in deionized H_2O. The fractions were collected and purified by ion exchange high performance liquid chromatography using a Whatman Partisil-SAX column to provide pure β-L-Fd4CTP. [^{1}H]NMR analysis (300 MHz; CD_3OD) yielded these results: δ 7.96 (d, J = 9 Hz, 1H), 7.85 (m, 1H), 7.38 (m, 1H), 6.95 (s, 1H), 6.53 (d, J = 7.5 Hz, 1H), 5.89 (d, J = 7.5 Hz, 1H), 4.94 (m, 1H), 4.21 (m, 2H). Fast-atom bombardment-mass spectrometry was calculated for $C_9H_{13}O_{12}N_3P_3F$ 467; found, 465.

Inhibition of HIV RT and human DNA polymerases by β-L-Fd4CTP, β-L-FddCTP, and ddCTP. The incubation mixture (10 μl) contained a buffer optimal for each enzyme as described previously (Kukhanova *et al.*, 1995), 50 nM complex (M13mp19 phage DNA-22-mer oligonucleotide primer), 2 μM dCTP, 20 μM each of three other dNTPs, 0.5 μCi [α-^{32}P]dCTP, different amounts of dCTP analogs, and 0.5–1 unit of enzyme. One unit of activity was defined as the amount of enzyme that catalyzed the incorporation of 1 nmol of dTMP into activated DNA per hour at 37° for human DNA polymerases, and as the amount of HIV RT that incorporated 1 nmol of dTMP into the poly(rA)oligo(dT) complex. The reaction mixtures were initiated with enzyme and incubated at 37°; the rate of incorporation of [α-^{32}P]dCMP residues into DNA was linear with respect to time. After incubation, 8 μl of reaction mixture was spotted onto DE81 paper. The paper was washed with 0.5 M NH_4HCO_3, dried, and counted as described previously (Kukhanova *et al.*, 1995).

DNA primer extension assays. The reaction mixture (8 μl) for conducting the single nucleotide incorporation into the standing start point of DNA contained the optimal buffer, 25 nM 5′-[^{32}P]-primer annealed to M13mp19 phage DNA as above, and different amounts of dCTP analogs as shown in Fig. 4. The reactions were initiated by the addition of enzyme, and the incubation time of all experiments was chosen so that the yield of the reaction products was linear as a function of time. The reactions were terminated by adding 5 μl of formamide stop solution, and the reaction products were analyzed by autoradiography after separation on 15% polyacrylamide sequencing gels as described previously (Kukhanova *et al.*, 1995).

The bands of 23-mer on the X-ray film, which represent the incorporation of dCTP analogs into the standing start point of 3′-ends of 5′-[^{32}P]-primer were quantified with the aid of a densitometer (Molecular Dynamics, Sunnyvale, CA) as described previously (Mendelman *et al.*; 1990, Kukhanova *et al.*, 1995). The bands were scanned, and the percentage of the primer that was converted into product per unit time was taken as the relative reaction rate. The K_m and V_{max} values were calculated from a double reciprocal plot of initial rate versus concentration of dCTP analog, at a fixed primer-template concentration (Boosalis *et al.,* 1989). The incubation time for all experiments was chosen to produce a linear dependence between yield of product and time. The utilization of primer was usually less than 30%.

Chain-termination sequencing reactions. A modification of the dideoxy-chain termination sequencing procedure (Sanger *et al.*, 1977) was used to assay β-L-Fd4CTP for base-specific chain termination. Reaction mixtures (8 μl) contained buffer; 25 nM 5′-[^{32}P]-primer-template complex as above; 20 μM each dATP, dTTP, and dGTP; 1 μM dCTP; and 1 unit of HIV RT or 2 units of pol γ. Additionally, the reaction mixtures contained β-L-Fd4CTP: 2 μM, 5 μM, and 20 μM, when reactions were catalyzed with pol γ and 0.25 μM, 0.5 μM, and 2 μM when catalysis was provided by HIV RT. As a control, DNA sequence analysis was performed using ddNTPs as chain terminators. After incubation at 37° for 30 min, the reaction mixtures were chased for 30 min with an additional 2 μl of a solution containing 250 μM of all four natural dNTPs. The reaction products were analyzed by 12% polyacrylamide gel electrophoresis as described above.

Results

Synthesis of β-L-Fd4CTP. Because of the acid instability of the 2′,3′ double bond in β-L-Fd4C, many conventional methods used for the 5′-phosphorylation have failed to provide the desired β-L-Fd4CTP (Hoard and Ott, 1965). After many unsuccessful attempts, because of the base-mediated hydrolysis of the neutral tributylammonium (*S*-acyl-2-thioethyl)-bearing monophosphate prodrug of β-L-Fd4C, we finally succeeded in the synthesis of β-L-Fd4CTP using the one-pot procedure outlined in Fig. 1. In this case, 2-cyanoethyl diisopropyl chlorophosphoramidite was employed as the 5′-monophosphorylating agent (Beancage, 1993). [^{1}H]NMR characteristics of the product are described in Materials and Methods. The retention time of β-L-Fd4CTP on an anion exchange high performance liquid chromatography column was 19.2 min, which is the same retention time as the [^{3}H]β-L-Fd4CTP obtained by enzymatic synthesis.

Inhibition of HIV RT and human DNA polymerases by β-L-Fd4CTP, β-L-FddCTP, and ddCTP. The structure of β-L-Fd4CTP is shown in Fig. 2. The inhibition pattern of HIV RT and human DNA polymerases by β-L-Fd4CTP was compared with that by ddCTP and β-L-FddCTP with the use of the complex of a 22-mer oligonucleotide primer annealed to M13mp19 phage DNA (Fig. 3). β-L-Fd4CTP was about 4-fold more potent as an inhibitor than ddCTP for HIV RT, and

8–10-fold more effective an inhibitor than β-L-FddCTP. β-L-Fd4CTP effectively inhibited pol γ, but in contrast to HIV RT, the efficiency of inhibition was about one order of magnitude lower than that of ddCTP. The inhibitory effect of β-L-Fd4CTP on pol β was twice that of ddCTP. The concentrations of β-L-Fd4CTP that inhibited the yield of products by 50% were 0.20 ± 0.05 μM, 1.8 ± 0.5 μM, and 4 ± 1 μM for HIV RT, pol γ, and pol β, respectively. By comparison, the K_i values of 5′-triphosphate of 3TC have been reported as 1 μM, 0.01 μM, and 1.2 μM for HIV RT, pol γ, and pol β, respectively (Chang *et al.*, 1992). β-L-Fd4CTP at a concentration as high as 40 μM had no inhibitory effect on pol ϵ, but inhibited pol α by 10–20% at 20 μM. The effects of ddCTP and β-L-FddCTP on pol α was in accordance with those previously described (Copeland *et al.*, 1992; Kukhanova *et al.*, 1995).

Kinetic parameters of the incorporation of β-L-Fd4CTP into the standing start point of DNA chain. The β-L-FddCTP and β-L-Fd4CTP were evaluated for their incorporation into the standing start point of DNA by HIV RT and human DNA polymerases, in a system containing M13mp19 phage DNA annealed with 5′-[^{32}P]-22-mer oligonucleotide primer. Although a large body of experimental data exists on steady state kinetic constants of HIV RT and human DNA polymerases with respect to ddCTP and β-L-FddCTP, these data were generated not only under different conditions, but more importantly, with different template-primers (St. Clair *et al.*, 1987; Ono *et al.*, 1989; Eriksson *et al.*, 1995; Kukhanova *et al.*, 1995; Wilson *et al.*, 1996; Ueno and Mitsuya., 1997). Steady state kinetic constants are known to be greatly influenced by the template complex and salt concentration (Beard and Wilson, 1993). We therefore compared the K_m and V_{max} values of HIV RT and human DNA polymerases with respect to ddCTP, β-L-FddCTP, and β-L-Fd4CTP using the same template and a buffer optimal for each enzyme. Fig. 4 illustrates the dose dependence of the incorporation of β-L-Fd4CTP into the standing start point of 5′-[^{32}P]-primer by HIV RT, pol γ, pol β, pol α, and pol ϵ. The bands corresponding to the 23-mer primer were scanned with a densitometer, and the K_m and relative V_{max} values were estimated in the concentration ranges shown in Fig. 4. Similar methods were applied for estimation of the K_m and V_{max} values of ddCTP and β-L-FddCTP. The elongation of the primer with β-L-Fd4CTP by HIV RT and DNA polymerases in the standing start point was saturated at 0.25 μM-0.5 μM for HIV RT, 1 μM-2 μM for pol γ, and 5 μM for pol β. Table 1 shows the K_m and V_{max} values for all analogs, and the ratio of the V_{max} to K_m reflects the efficiency of the analogs' incorporation into the standing start point. The behavior of HIV RT and pol γ in respect to all three analogs was similar. Both HIV RT and pol γ were able to use all three analogs, but β-L-Fd4CTP was the most effective substrate for HIV RT. Its incorporation efficiency was 4-fold higher than that of ddCTP, which had been the most potent nucleotide inhibitor of HIV RT discovered previously. The β-L-Fd4CTP is also a good substrate for pol γ and pol β, but in contrast to HIV RT, its incorporation into DNA by pol γ was 7–8-fold lower than that of ddCTP, and about 3-fold lower than by pol β. Pol α could use β-L-Fd4CTP as a substrate, but only at high concentration (>40 μM) (Fig. 4). As a consequence, a direct estimation of the K_m value could not be achieved for this enzyme. It should be mentioned that ddCTP or β-L-FddCTP up to a concentration of 100 μM could not be incorporated into DNA by pol α (data not shown). These data excluded possible contamination of pol α samples with pol γ or pol β, and correlated with findings reported previously (Copeland *et al.*, 1992). All steady state kinetic values presented here must be regarded as apparent because a saturating primer-template concentration was not achieved in these experiments. Another complicating factor in estimation of the K_m values for pol γ was the 3′->5′ exonuclease activity of this enzyme, which could degrade primers terminated by different analogs at different rates. To overcome these problems, we deter-

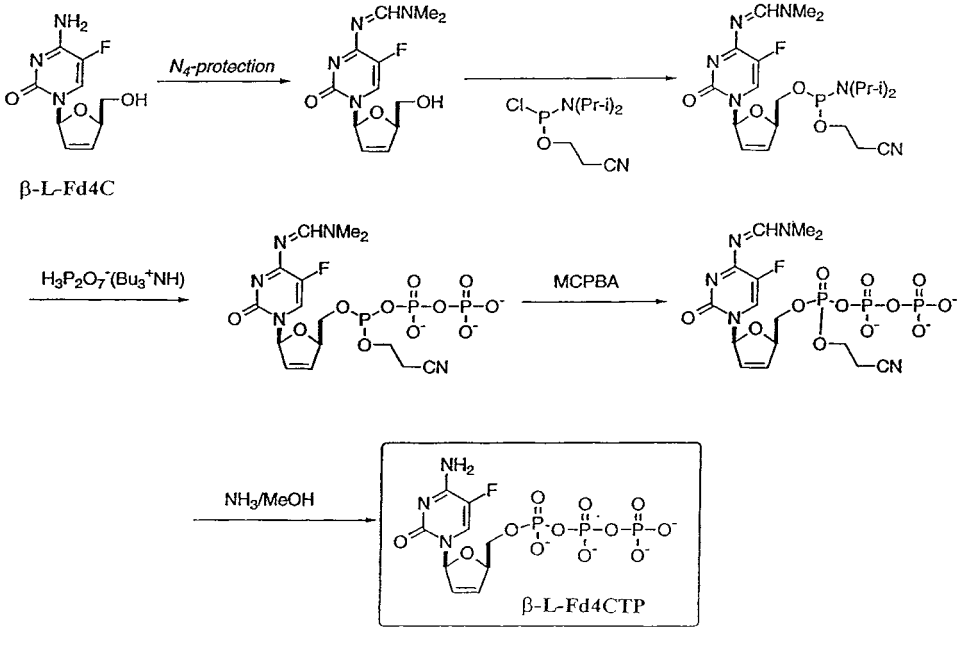

Fig. 1. Outline of one-pot procedure used for successful synthesis of β-L-Fd4CTP.

mined the relative efficiency of HIV RT or DNA polymerases in incorporating different dCTP analogs. Comparison was made mainly with ddCTP, which is the most effective inhibitor of HIV RT and pol γ. Inhibition of pol γ subsequently leads to delayed cellular toxicity (Chen and Cheng, 1989).

Chain-termination of DNA synthesis by β-L-Fd4CTP. The competition of β-L-Fd4CTP with dCTP for incorporation into the growing DNA strand by HIV-1 or pol γ was assayed using the modified dideoxy-chain termination sequencing procedure described previously (Sanger *et al.*, 1977). The same primer-template as above was used in these experiments (Fig. 5). In the absence of β-L-Fd4CTP and in the presence of all four natural dNTPs, the synthesis proceeded up to 70 nucleotides (Fig. 5A, *lane 8*). It should be noted that some natural stop sites at positions C28 and G38–40 were observed when the reaction mixture contained all four natural dNTPs. Fig. 5, *lanes 1–4*, shows the sequence of primer extension using ddNTPs as chain-terminators and pol γ or HIV RT (Fig. 5, A and B). As the concentrations of β-L-Fd4CTP were increased from 0.25 μM to 2 μM for HIV RT, and from 2 μM to 20 μM for pol γ, more and more DNA fragments terminated at C-sites were observed (C23, C27, C29, C34–36. C43–44). These observations indicate that β-L-Fd4CMP residues were incorporated into the DNA chain in place of dCMP. It is noteworthy that the concentration of β-L-Fd4CTP that produced the DNA sequencing pattern with HIV RT was 8–10-fold less than that observed with pol γ. This result shows that β-L-Fd4CTP can compete with dCTP for incorporation when HIV RT or pol γ are used as the enzymes. This observation is supported by the data presented in Table 1 and Figs. 3 and 4.

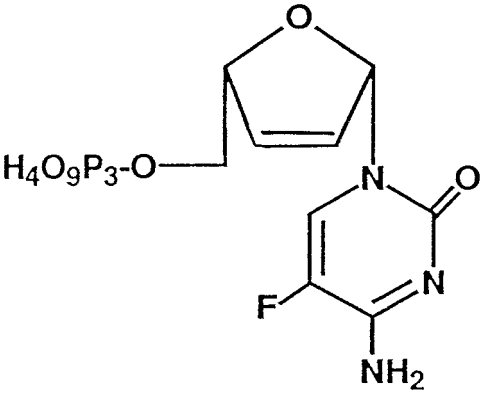

Fig. 2. Structure of β-L-Fd4CTP.

Discussion

Considerable effort has been directed toward the search for a novel nucleoside structure among L-nucleoside analogs for use as an anti-HIV and anti-HBV agent. Recently, a novel 2′,3′-unsaturated L-nucleoside derivative, β-L-Fd4C, was found to be the most active agent against HBV, and also to exhibit significant anti-HIV activity in cell culture (Lin *et al.*, 1996; Faray *et al.*, 1997). The cell growth inhibitory activity of this compound against CEM cell lines was about the same as that of ddC and was 10-fold higher than that of β-L-FddC. Its ability to inhibit mitochondrial DNA synthesis was much less than that of ddC (Lin *et al.*, 1996). The mechanism of its

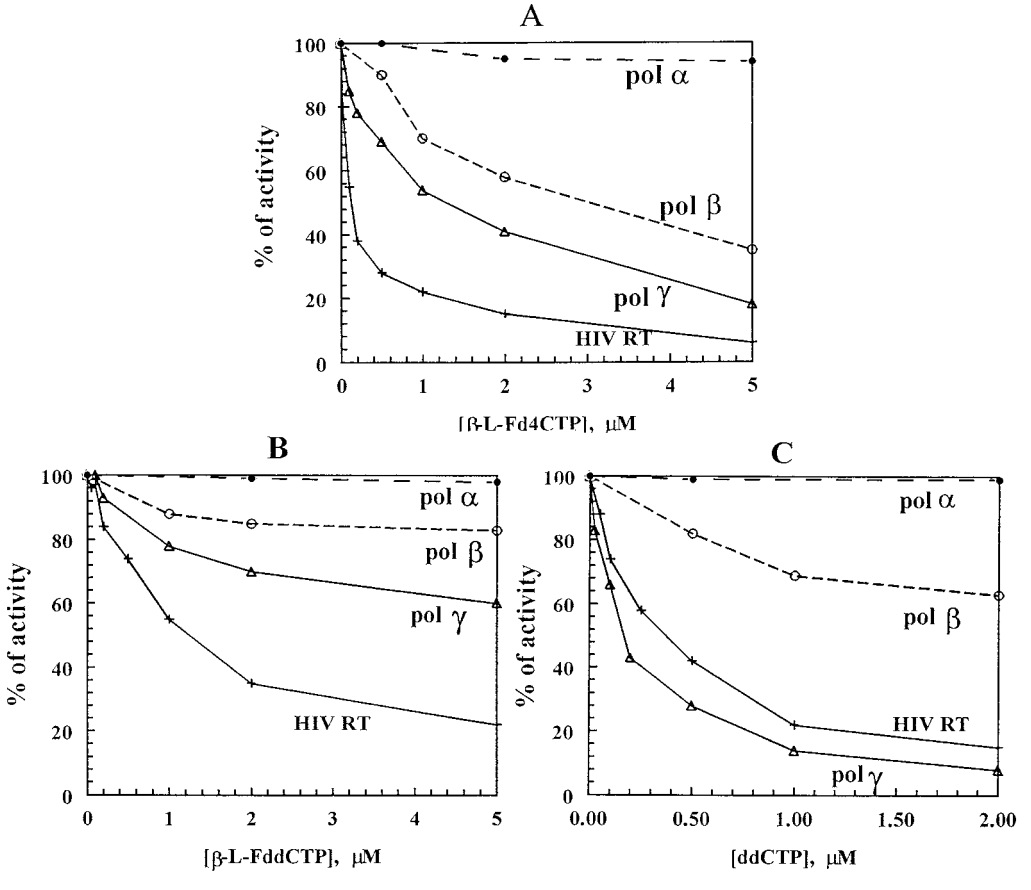

Fig. 3. Inhibition of M13mp19 phage DNA synthesis catalyzed by HIV RT or human pol γ, pol β, or pol α with A, β-L-Fd4CTP; B, β-L-Fd-dCTP; and C, ddCTP. The assay conditions are described in Materials and Methods. The incorporation of [α-^{32}P]dCTP into primer-template without inhibitors was taken as 100%. *Points*, average of three experiments, with duplicates performed in each experiment.

antiviral and cell growth inhibition activities may be due to the effect of its 5′-triphosphate metabolite on viral and human DNA polymerases. Recently, the formation of 5′-triphosphate metabolites in cells treated with β-L-Fd4C has been demonstrated (Dutschman *et al.*, 1998). The efficiency of formation of the phosphorylated metabolites of β-L-Fd4C was higher than that of β-L-FddC and 3TC in cell culture. Whether the more potent anti-HIV action of β-L-Fd4C relative to that of β-L-FddC is caused by the different affinity of their 5′-triphosphate metabolites for HIV RT is not clear.

This work has demonstrated that β-L-Fd4CTP is a more potent inhibitor of HIV RT than β-L-FddCTP is. The inhibitory potency is correlated with the efficiency of β-L-Fd4CTP and β-L-FddCTP as a substrate for HIV RT (Fig. 3, Table 1). β-L-Fd4CTP is a good substrate for pol γ and pol β, but it is a better substrate for HIV RT. The K_i value of β-L-Fd4CTP has been reported as 5-fold less than that of 5′-triphosphate of 3TC (Chang *et al.*, 1992). The high affinity of β-L-Fd4CTP for HIV RT may be caused by a preferable recognition of the2′,3′-dideoxy-2′,3′-didehydro structure of the ribose residue compared with that of the 2′,3′-dideoxy structure by HIV RT.

The X-ray analysis of β-D-2′,3′-dideoxy-2′,3′-didehydronucleosides showed that the 2′,3′ double bond limits the conformational flexibility of the sugar ring. In contrast to the deoxyribose ring, the didehydrofurane ring is nearly planar with O(4′) being slightly above the plane of the other four atoms (*endo*-configuration) (Birnbaum *et al.*, 1989; Harte *et al.*, 1991; Van Roey and Chu, 1992). It has been suggested that such a planar conformation of the ribose residue may represent a transition state of the substrates of HIV RT and some cellular kinases (Harte *et al.*, 1991; Krayevsky and Watanabe, 1993). It has been shown that natural dNTPs in a complex with *Escherichia coli* DNA polymerase I undergo conformational rearrangement resulting in significant flattening of the deoxyribose residue (Ferrin and Mildvan, 1985, 1986). It is logical to predict that the conformation of the ribose residue in L- and D-enantiomers of unsaturated nucleosides is similar, and flattened conformation might mimic a transition state of β-L-Fd4CTP during enzymatic reaction and facilitate its binding to HIV RT. This hypothesis is consistent with the observations made by others, that both β-L- and β-D-enantiomers of d4NTP were more effective inhibitors of HIV RT than were β-L-ddNTP and β-D-ddNTP, respectively (Ono *et al.*, 1989; Wilson *et al.*, 1996). Our results do not prove that a flattened conformation of the ribose residue

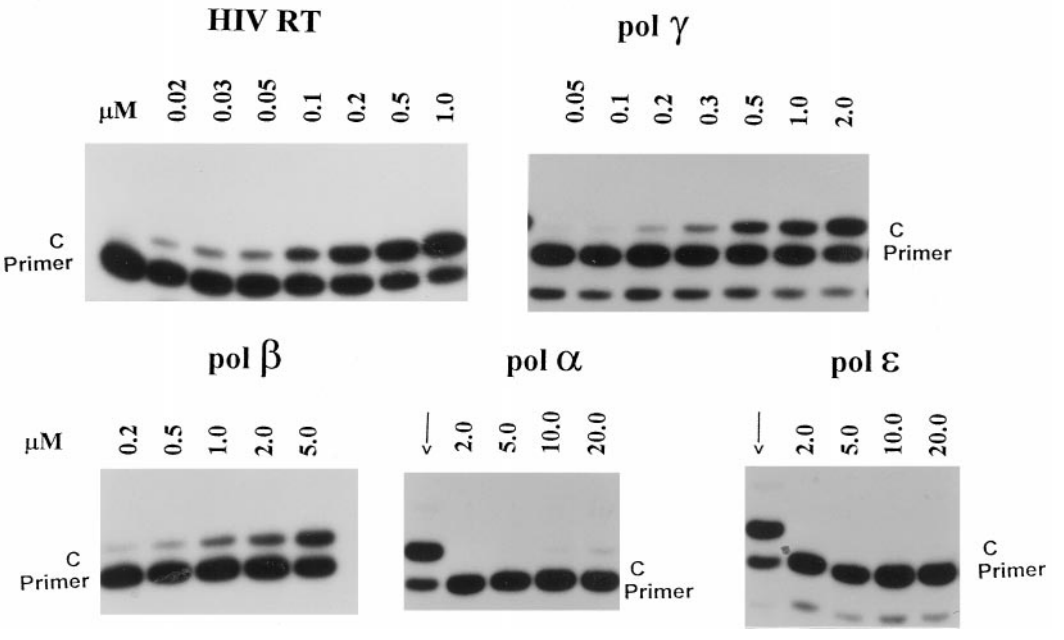

Fig. 4. Gel analysis of the capacity of HIV RT, pol γ, pol β, pol α, and pol ϵ to incorporate β-L-Fd4CTP into the standing start point of 5′-[^{32}P]-22 mer oligonucleotide primer annealed with M13mp19 phage DNA. Assays were performed as described in Materials and Methods. The concentrations of β-L-Fd4CTP used for each enzyme are shown. Arrows indicate the incorporation of dCTP (0.5 μM). *Bands under position of primer*, 3′->5′ exonuclease activity of pol γ and pol ϵ.

TABLE 1

Apparent kinetic constants of interaction of ddCTP, β-L-FddCTP, and β-L-Fd4CTP with standing start point of primer-template

Values and standard deviations are from one experiment repeated at least three times with very close results. The K_m and V_{max} were calculated as described under Materials and Methods.

DNA polymerase		dCTP analogs		
		ddCTP	β-L-FddCTP	β-L-Fd4CTP
HIV RT	K_m, μM	0.35 ± 0.10	0.45 ± 0.15	0.076 ± 0.010
	V_{max}	0.85 ± 0.08	0.30 ± 0.08	0.68 ± 0.08
	V_{max}/K_m	2.4	0.66	8.9
Pol γ	K_m, μM	0.13 ± 0.05	1.6 ± 0.6	0.23 ± 0.07
	V_{max}	1.6 ± 0.5	0.64 ± 0.5	0.40 ± 0.05
	V_{max}/K_m	12	0.43	1.7
Pol β	K_m, μM	0.16 ± 0.05	0.32 ± 0.08	0.19 ± 0.07
	V_{max}	0.7 ± 0.2	0.12 ± 0.03	0.21 ± 0.05
	V_{max}/K_m	4.4	0.39	1.2
Pol α	K_m, μM	>50	>50	>20
Pol ϵ	K_m, μM	>50	>50	>50

of nucleosides is the only requirement for their potent anti-HIV activity, but our findings do show that a flattened conformation of the ribose residue has a significant effect on the interaction of their 5′-triphosphates with HIV RT.

A high activity of unsaturated nucleoside analogs in cell culture indicates also that these compounds are substrates for all steps of phosphorylation. Whatever the mechanism, these results may be important in the design of anti-HIV compounds. Although pol γ showed behavior similar to that of HIV RT toward some dNTP analogs (Eriksson *et al.*, 1995), β-L-Fd4CTP was a much less potent substrate for pol γ than for HIV RT. It should be noted that compared with ddC, both β-L-FddC and β-L-Fd4C are much poorer inhibitors of mitochondrial DNA synthesis in cell culture (Lin *et al.*, 1996; Dutschman *et al.,* submitted for publication). The lack of potent inhibition of mitochondrial DNA synthesis in cell culture by β-L-Fd4C could be caused by either less efficiency of the interaction of its 5′-triphosphate metabolites with pol γ, or an insufficient amount of the 5′-triphosphate metabolite in the mitochondria. This question is under investigation. β-L-Fd4CTP is not a substrate for pol ϵ, and is a very poor substrate for pol α. Its incorporation into DNA was detected only at a high concentration of β-L-Fd4CTP in the primer extension assay (Fig. 4). The ability of β-L-Fd4CTP to be a substrate for pol α could account for its inhibition of cell growth. Other possible mechanisms of the growth inhibitory action of β-L-Fd4C, unrelated to interaction of its 5′-triphosphate with pol α, are under current investigation.

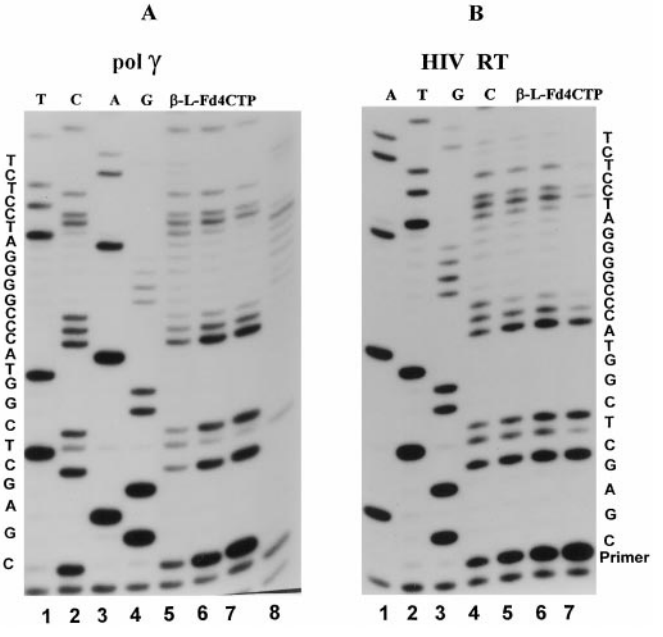

Fig. 5. Gel analysis of the chain terminating sequencing reaction with β-L-Fd4CTP with the use of A, pol γ, or B, HIV RT. The letters on both sides of the figure indicate the sequence of the growing DNA chain after the primer. A, *lane 8*, shows the DNA synthesis catalyzed by pol γ without chain terminators. A and B, *lanes 1–4*, DNA sequence analysis using ddNTPs as chain terminators and A, pol γ, or B, HIV RT, respectively. A and B, *lanes 5–7*, DNA sequencing analysis using β-L-Fd4CTP as the chain terminator at, in A, 2 μM, 5 μM, and 20 μM, respectively, and, in B, 0.25 μM, 0.5 μM, and 2 μM, respectively. Reaction conditions are described in Materials and Methods.

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