

The 5'-Triphosphates of the (-) and (+) Enantiomers of *cis*-5-Fluoro-1-[2-(Hydroxymethyl)-1,3-Oxathiolane-5-yl]Cytosine Equally Inhibit Human Immunodeficiency Virus Type 1 Reverse Transcriptase

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The (-) enantiomer of *cis*-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolane-5-yl]cytosine is a potent inhibitor of human immunodeficiency virus type 1 in vitro. The 5'-triphosphates of the (-) and (+) enantiomers equally inhibited the production of full-length minus-strand DNA in an endogenous reverse transcriptase reaction, each competitively inhibited DNA synthesis, and each was used as a chain-terminating substrate.

cis-5-Fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolane-5-yl]cytosine (FTC) has been reported to have potent antiviral activity against human immunodeficiency virus type 1 (HIV-1) in vitro (10, 11). Because of different toxicities, the (+) and (-) enantiomers of FTC have been resolved and the activity of each has been studied. (-)-FTC, which has a 1- β -L configuration (13), is significantly more active than its (+) enantiomer, which has the natural 1- β -D configuration, against HIV-1 in cellular assays. Part of this selectivity is accounted for by the fact that (-)-FTC is phosphorylated to a greater extent than is (+)-FTC (10); (-)-FTC is a better substrate for deoxycytidine kinase and deoxycytidine monophosphate kinase than is (+)-FTC (3). In the study described here, we examined the inhibition of HIV-1 reverse transcriptase (RT) by each enantiomer of FTC 5'-triphosphate (FTCTP) to determine whether at the level of DNA synthesis there is differential recognition of the two compounds. The results indicated no significant difference in the 50% inhibitory concentrations (IC₅₀s) of the two enantiomers, which were determined in an endogenous RT assay with isolated, permeabilized virus. Under steady-state assay conditions with purified RT, both (-)- and (+)-FTCTP equally inhibited RNA- and DNA-directed DNA syntheses. Additionally, both enantiomers of FTC were used as chain-terminating substrates.

Endogenous HIV-1 reaction. In the endogenous reaction, RT catalyzes the synthesis of genomic minus-strand DNA in the purified viral particle by using exogenously supplied deoxynucleoside triphosphates (dNTPs) and buffers that diffuse into the viral core. The advantage of this system is that specific inhibition of the RT can be studied in an intact viral core, simulating the process of DNA synthesis during infection. Inhibition of the endogenous reaction was studied with (-)- and (+)-FTCTP and compared with those of ddCTP and zidovudine triphosphate (AZTTP). Both (-)-FTCTP and (+)-FTCTP were synthesized by the method of White et al. (14) as reported previously (3, 10) and were >97% pure as judged by high-pressure liquid chromatogra-

phy ($\lambda_{\max} = 289$ in 0.1 N HCl; $\epsilon = 8,168 \text{ cm}^{-1} \text{ M}^{-1}$). Purified HIV-1 strain IIB virions were purchased from Universal Biotechnology Inc., Rockville, Md., for use in the endogenous reaction. Reactions were carried out as described by Borroto-Esoda and Boone (1). A typical reaction consisted of 30 μl of a solution containing 10 μg of virion protein, 100 mM Tris HCl (pH 8.1), 15 mM NaCl, 3 mM MgCl₂, 0.5 mM (each) dNTP, 1 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 0.1% Nonidet P-40, and inhibitor at specified concentrations (Fig. 1). The reaction mixtures were incubated for 2 h at 39°C. The DNA products were then extracted with phenol-chloroform, precipitated with ethanol, and analyzed by hybridization with an RNA probe specific for HIV-1 minus-strand DNA. The DNA products were quantitated by using a Molecular Dynamics Phosphor Imager. Figure 1 shows the inhibition of minus-strand DNA production by ddCTP, (-)-FTCTP, and (+)-FTCTP. No significant selectivity between the two enantiomers was observed. Both inhibited full-length product formation by 50% at concentrations similar to that obtained with ddCTP, with values being $0.32 \pm 0.15 \mu\text{M}$ for (-)-FTCTP, $0.45 \pm 0.09 \mu\text{M}$ for (+)-FTCTP, and $0.28 \pm 0.06 \mu\text{M}$ for ddCTP. The three cytidine analogs did not inhibit the reaction as well as AZTTP did, which had an IC₅₀ of $0.10 \pm 0.01 \mu\text{M}$. Values are means \pm standard deviations.

Enzyme assays. To determine whether both enantiomers were competitive inhibitors of RT, steady-state kinetic analysis was performed on isolated, purified enzyme. HIV-1 RT was purified as described previously from *Escherichia coli* expressing HXB2 HIV-1 p66 RT (6). All enzyme assays were performed at 37°C as described previously (5). AZTTP was kindly provided by Wayne Miller (Burroughs Wellcome) (8). Both (-)-FTCTP and (+)-FTCTP were synthesized by the method of White et al. (14). By using the homopolymeric template-primer poly(rI) · p(dC)₁₉₋₂₄, linear competitive inhibition was observed for both (-)-FTCTP and (+)-FTCTP during RNA-dependent DNA synthesis (Fig. 2). The slope and K_m (apparent) replots indicated pure competitive inhibition with respect to dCTP for each enantiomer. K_i values of 0.90 ± 0.04 and $2.5 \pm 0.4 \mu\text{M}$, respectively, for the (-) and the (+) enantiomers differed only by a factor of 2 (Table 1).

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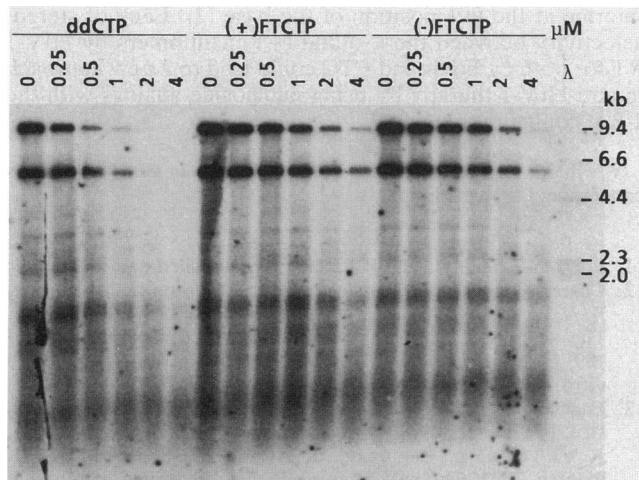


FIG. 1. Inhibition of the HIV-1 endogenous reaction. Reactions were performed as described in the text.

Both (-)-FTCTP and (+)-FTCTP inhibited dCTP turnover somewhat better than did ddCTP, which had a K_i of $5.1 \pm 0.7 \mu\text{M}$. Assays for AZTTP and ddTTP were carried out with poly(rA) · p(dT)₁₀ as the template-primer. AZTTP inhibited the reaction, with a K_i value similar to that observed for ddTTP (0.016 ± 0.001 and $0.022 \pm 0.005 \mu\text{M}$, respectively).

To compare each of the inhibitors on the same template-primer during DNA-directed DNA synthesis, DNA-primed heteropolymeric DNA mp18HXBRT was constructed (6). This DNA-primed DNA has several advantages over the homopolymeric template-primers studied previously. Primarily, the insertion of the 1.7-kb HIV-1 RT-coding region into the M13 mp18 vector and the priming in this region enabled us to study RT synthesis on authentic HIV-1 DNA. Additionally, all nucleoside analog inhibitors could be analyzed by using the same system, which permits direct comparisons of K_i values for different dNTP analogs. Saturating concentrations of template-primer and dNTPs were found to be $30 \mu\text{g/ml}$ and $25 \mu\text{M}$, respectively. K_m values

TABLE 1. K_i values for HIV-1 RT

Compound	K_i (μM)		
	poly(rI) · p(dC) ₁₉₋₂₄ ^a	mp18HXBRT ^b	poly(rA) · p(dT) ₁₀ ^c
(-)-FTCTP	0.90 ± 0.04	4.7 ± 0.9	NA ^d
(+)-FTCTP	2.5 ± 0.4	1.7 ± 0.4	NA
ddCTP	5.1 ± 0.7	0.80 ± 0.17	NA
AZTTP	NA	0.33 ± 0.03	0.016 ± 0.001
ddTTP	NA	0.50 ± 0.06	0.022 ± 0.005

^a The concentration of poly(rI) · p(dC)₁₉₋₂₄ was $2 \mu\text{M}$.

^b Primer DNA, 22-mer, was annealed to the single-stranded mp18 vector which contained the entire 1.7-kb HIV-1 RT coding region inserted into the *Eco*RI and *Hind*III sites of the polylinker as described in the text. The concentration was $30 \mu\text{g/ml}$. Reactions for AZTTP and ddTTP contained $25 \mu\text{M}$ (each) dATP, dCTP, dGTP and $10 \mu\text{M}$ [³H]dTTP. Reactions for (-)-FTCTP, (+)-FTCTP, and ddCTP contained $25 \mu\text{M}$ (each) dATP, dGTP, dTTP and $5 \mu\text{M}$ [³H]dCTP.

^c Concentration was $0.5 \mu\text{M}$. The reaction conditions were as described in the text.

^d NA, not applicable.

determined for dCTP and dTTP were 1.0 ± 0.1 and $3.1 \pm 0.8 \mu\text{M}$, respectively, and the k_{cat} values were found to be 0.07 ± 0.01 and $0.24 \pm 0.03 \text{ s}^{-1}$, respectively. The k_{cat}/K_m values for the two dNTPs were similar (0.07 and $0.08 \text{ s}^{-1} \mu\text{M}^{-1}$, respectively). The K_i values determined with mp18HXBRT as the template-primer were calculated from Dixon plot analysis for a competitive inhibitor. Of the inhibitors studied, AZTTP was the most potent on mp18HXBRT, with a K_i value of $0.33 \mu\text{M}$ (Table 1). (+)-FTCTP appeared to be a slightly better inhibitor than (-)-FTCTP during DNA-directed DNA synthesis, with K_i values of 1.7 ± 0.4 and $4.7 \pm 0.9 \mu\text{M}$, respectively. The two enantiomers of FTCTP did not inhibit dCTP turnover as well as ddCTP did, which had a K_i value two- to sixfold lower than those of the oxathiolane analogs.

Chain-termination substrate assays. Both (-)-FTCTP and (+)-FTCTP were analyzed for their chain-terminating activities. The compounds were assayed on DNA-primed mp18HXBRT by a modification of the chain-termination sequencing procedure of Sanger et al. (9) (Fig. 3). For comparison, ddATP, ddCTP, ddGTP, ddTTP, and AZTTP

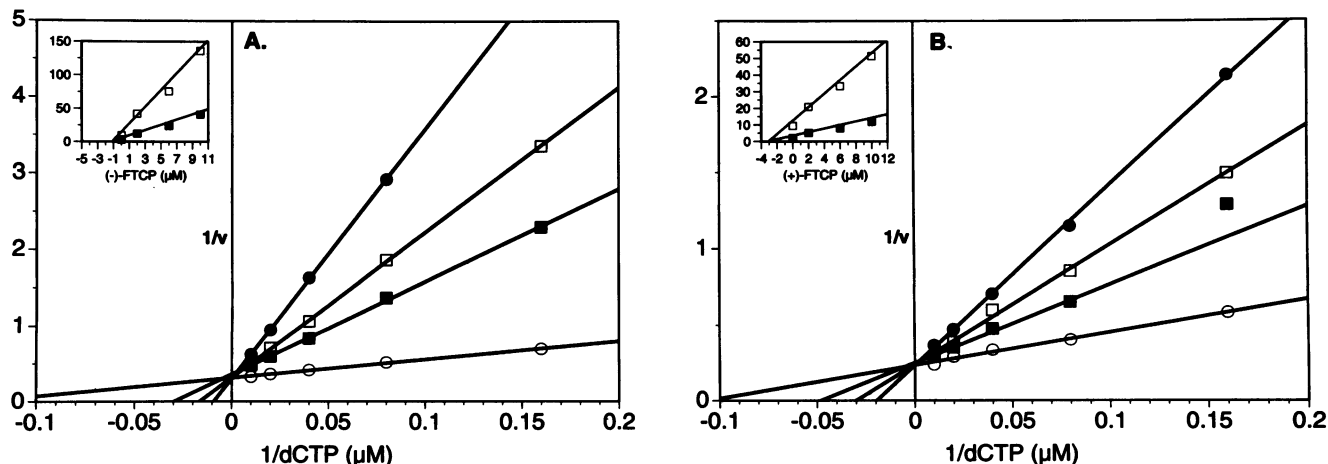


FIG. 2. Inhibition of HIV-1 RT by (-)-FTCTP and (+)-FTCTP. (A) Lineweaver-Burk plot of (-)-FTCTP inhibition of [³H]dCTP turnover on poly(rI) · p(dC)₁₉₋₂₄. (Inset) Slope (■) and $K_m(\text{app})$ (□) replot versus (-)-FTCTP concentrations. (B) Lineweaver-Burk plot of (+)-FTCTP inhibition of [³H]dCTP turnover on poly(rI) · p(dC)₁₉₋₂₄. (Inset) Slope (■) and $K_m(\text{app})$ (□) replot versus (+)-FTCTP concentrations. Inhibitor concentrations were $10 \mu\text{M}$ (●), $6 \mu\text{M}$ (□), $2 \mu\text{M}$ (■), and no inhibitor (○).

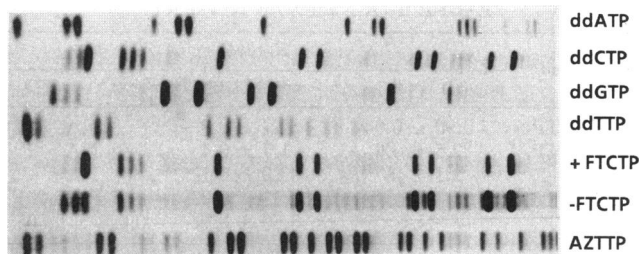


FIG. 3. Autoradiograph of chain-terminating sequencing reactions with dNTP analogs using HIV-1 RT. Concentrations of all chain-terminating substrates were 1 μ M. Reactions were performed on mp18HXBRT as described in the text.

are also shown in Fig. 3. Synthesis was performed in the presence of [35 S]dATP to enable visualization of newly synthesized DNA chains. The reaction mixtures used in the ddCTP, (+)-FTCTP, and (-)-FTCTP termination reactions contained 20 mM Tris (pH 8.0), 10 mM MgCl₂, 25 mM KCl, 40 μ M dGTP, 40 μ M dTTP, 2 μ M dCTP, 2 μ l of preannealed mp18HXBRT, 6 μ Ci of [35 S]dATP, and 80 ng of HIV-1 RT in a total volume of 7 μ l. Reaction mixtures in the ddTTP and AZTTP termination reactions contained the same components as described above, with the exception that the dNTPs were present at the following concentrations: 40 μ M dCTP, 40 μ M dGTP, and 2 μ M dTTP. Additionally, reaction mixtures contained 1 μ M either ddCTP, ddATP, ddGTP, ddTTP, AZTTP, (+)-FTCTP, or (-)-FTCTP, as indicated in Fig. 3. The 7- μ l reaction mixtures were incubated for 30 min at 37°C and were then chased for 30 min at 37°C with an additional 2.5 μ l of a solution containing 250 μ M dATP, dCTP, dGTP, and dTTP and 40 ng of HIV-1 RT. The reactions were terminated by the addition of 4 μ l of formamide stop solution, and the DNA reaction products were analyzed by using autoradiographs of wedge polyacrylamide sequencing gels. As described previously (12), AZTTP exhibits termination at positions similar to that exhibited by ddTTP. The primer extension products of both the (-) and (+) enantiomers of FTCTP were similar to the products observed for ddCTP, indicating that they were incorporated into DNA strands at cytosine residue sites. Some additional stops corresponding to ddATP and ddTTP were seen with (-)-FTCTP that were not seen with either (+)-FTCTP or ddCTP, which may reflect the lack of specificity of RT specifically with the (-) enantiomer and which may contribute to the antiviral activity.

In conclusion, both the (+) and the (-) enantiomers of FTCTP are potent inhibitors of HIV-1 RT. Each enantiomer inhibited full-length genomic minus-strand DNA production in the HIV-1 endogenous reaction, each was a competitive inhibitor of the RT in steady-state enzyme assays, and each was a chain-terminating substrate. Differential antiviral activity appears to be due to differential metabolism and catabolism (3, 10) and not to the enantioselectivity by the RT. The lack of enantioselectivity by the RT with respect to (+)- and (-)-FTCTP is in good agreement with the results presented by Miller et al. (7), demonstrating that the RT is not preferentially inhibited by the 5'-triphosphate of either of the two enantiomers of carbovir. However, Hart et al. (4), in a study comparing the 5'-triphosphates of the (+) and (-) enantiomers of BCH189, concluded that the (+) enantiomer is more potent than the (-) enantiomer, 3'-thiacytosine-5'-triphosphate, in inhibiting the RT. These results are surprising, considering that BCH189 and FTC differ only by a

fluorine at the five position of the base (2). Lack of stereoselectivity between the (-) and (+) enantiomers by HIV-1 RT for both carbovir and FTC could lead to a new approach in anti-HIV-1 therapy by using nucleoside analogs with the 1- β -L configuration.

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