ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Oct. 1994, p. 2300–2305
0066-4804/94/$04.00+0

Vol. 38, No. 10

Inhibition of Human Immunodeficiency Virus Type 1 Reverse Transcriptase by the 5′-Triphosphate β Enantiomers of Cytidine Analogs

ABDESSLEM FARAJ,[1] LUIGI A. AGROFOGLIO,[1] JOHN K. WAKEFIELD,[2] SYLVIA McPHERSON,[2] CASEY D. MORROW,[2] GILLES GOSSELIN,[3] CHRISTOPHE MATHE,[3] JEAN-LOUIS IMBACH,[3] RAYMOND F. SCHINAZI,[4,5], AND JEAN-PIERRE SOMMADOSSI[1]*

Department of Pharmacology, Center for AIDS Research, The Comprehensive Cancer Center, and Division of Clinical Pharmacology,[1] and Center for AIDS Research, Department of Microbiology,[2] University of Alabama at Birmingham, Birmingham, Alabama 35294; Laboratoire de Chimie Bioorganique, URA 488 Centre National de la Recherche Scientifique, University of Montpellier II, 34095 Montpellier, France[3]; Veterans Affairs Medical Center, Decatur, Georgia 30033[4]; and Laboratory of Biochemical Pharmacology, Department of Pediatrics, School of Medicine, Emory University, Atlanta, Georgia 30033[5]

Received 6 May 1994/Returned for modification 10 June 1994/Accepted 26 July 1994

(−)-β-L-2′,3′-Dideoxycytidine (L-ddC) and (−)-β-L-2′,3′-dideoxy-5-fluorocytidine (L-FddC) have been reported to be potent and selective inhibitors of human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) in vitro. In the present study, the 5′-triphosphates of L-ddC (L-ddCTP) and L-FddC (L-FddCTP) were demonstrated to competitively inhibit HIV-1 reverse transcriptase (RT), with inhibition constants (K_is) of 2 and 1.6 μM, respectively, when a poly(rI) · oligo(dC)$_{10-15}$ template primer was used; in comparison K_i values for β-D-2′,3′-dideoxycytidine 5′-triphosphate (D-ddCTP) and β-D-2′,3′-dideoxy-5-fluorocytidine 5′-triphosphate (D-FddCTP) were 1.1 and 1.4 μM, respectively. Use of the mutant RT at position 184 (substitution of methionine to valine [M184V]), which is associated with resistance to β-L-2′,3′-dideoxy-3′-thiacytidine (3TC) and β-L-2′,3′-dideoxy-5-fluoro-3′-thiacytidine (FTC), resulted in significant increases (50- to 60-fold) in K_i values for L-ddCTP and L-FddCTP, whereas the elevation in K_i values for D-ddCTP and D-FddCTP was moderate (2-fold). L-ddCTP and L-FddCTP did not inhibit human DNA polymerases α and β up to 100 μM. In contrast, D-ddCTP and D-FddCTP inhibited human DNA polymerase β, with K_i values of 0.5 and 2.5 μM, respectively. By using sequencing analysis, L-ddCTP and L-FddCTP exhibited DNA chain-terminating activities toward the parental HIV-1 RT, whereas they were not a substrate for the mutant M184V HIV-1 RT. L-ddC and L-FddC did not inhibit the mitochondrial DNA content of human cells up to a concentration of 10 μM, whereas D-ddC and D-FddC decreased the mitochondrial DNA content by 90% at concentrations of 1 and 10 μM, respectively. All of these results suggest that further development of L-ddC, and L-FddC in particular, is warranted as a possible anti-HIV candidate.

(−)-β-L-2′,3′-Dideoxycytidine (L-ddC) and its 5-fluoro derivative (L-FddC) have been shown to have potent antiviral activities against human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) and human hepatitis B virus (HBV) in vitro (13, 14, 18, 22, 25). L-FddC potently inhibited HIV-1 and HIV-2 replication in vitro in various cell lines and exhibited a 90% effective concentration of 0.15 μM in human peripheral blood mononuclear cells infected with HIV-1 (14). Interestingly, L-FddC showed the highest selectivity index (SI; SI = 9,000) compared with those for L-ddC (SI = 37), β-D-2′,3′-dideoxy-5-fluorocytidine (D-FddC; SI = 35), and 3′-azido-3′-deoxythymidine (AZT; SI = 100) when the index was determined relative to that for toxicity to human bone marrow progenitor cells (14). L-ddC and L-FddC demonstrated a cross-resistance to HIV-1 variants resistant to 2′,3′-dideoxy-3′-thiacytidine (3TC; BCH-189) and its 5-fluoro-derivative (FTC) (14). Mutations of the reverse transcriptase (RT) at codon 184 from methionine (ATG, Met) to valine (GTG or GTA, Val) or isoleucine (ATA, Ile) have been generated through in vitro selection with 3TC or FTC (11, 12, 23). DNA sequence analysis of the RT gene amplified from a patient who had received 3TC therapy for 4 months revealed a mixture of the mutation at codon 184 from Met to Val (M184V) and the parental genotype, indicating that the mutation at the methionine at codon 184 (Met-184) can occur in vivo (23). In order to understand the mechanisms of action and drug resistance, the 5′-triphosphates of L-ddC (L-ddCTP) and L-FddC (L-FddCTP) were synthesized, and the inhibitory effects of these triphosphate derivatives toward HIV-1 RT recombinant wild-type (WT) (WT RT) and the site-directed mutagenesis recombinant mutant RT at position 184 (substitution of Met to Val [M184V]) were evaluated and compared with those of the natural (+)-β-D enantiomers 2′,3′-dideoxycytidine 5′-triphosphate (D-ddCTP) and 2′,3′-dideoxy-5-fluorocytidine 5′-triphosphate (D-FddCTP). The dideoxy-DNA chain termination sequencing procedure of Sanger et al. (20) was used to assess the DNA chain termination of L-ddCTP and L-FddCTP toward both the parental and mutant HIV-1 RTs. The selectivities of L-ddC and L-FddC were assessed by evaluating the effects of their triphosphate derivatives on human DNA polymerases α and β as well as the effects of these L-nucleosides on the mitochondrial DNA (mtDNA) content.

* Corresponding author. Mailing address: University of Alabama at Birmingham, Box 600, Volker Hall 113, University Station, Birmingham, AL 35294. Phone: (205) 934-8226. Fax: (205) 975-4871.

MATERIALS AND METHODS

Compounds. L-ddC and L-FddC were synthesized as described previously (13). D-FddC was kindly provided by V. Marquez (Laboratory of Medicinal Chemistry, National Institutes of Health, Bethesda, Md.). L-ddCTP, L-FddCTP, D-FddCTP, and 3TC 5′-triphosphate (3TCTP) were prepared by a standard phosphorylation method (28) from their corresponding nucleosides. The 5′-triphosphate derivatives were fully characterized by nuclear magnetic resonance (^1H,^{31}P), fast atom bombardment mass spectroscopy, high-performance liquid chromatography, and UV spectroscopy.

Materials. [^{3}H]dCTP (24 Ci/mmol) and [α-^{32}P]dATP (3,000 Ci/mmol) were purchased from ICN Biochemicals Inc. (Costa Mesa, Calif.). 2′,3′-Dideoxycytidine (ddC) and oligo(dC)$_{10-15}$ were purchased from Sigma Chemical Co. (St. Louis, Mo.). The Sequenase version 2.0 DNA sequencing kit and ddCTP were purchased from U.S. Biochemical Corp. (Cleveland, Ohio). Human DNA polymerases α and β were purchased from Molecular Biology Resources, Inc. (Milwaukee, Wis.). Calf thymus-activated DNA, poly(rI)$_n$, and M13mp18 plus-strand DNA were purchased from Pharmacia LKB Biotechnology (Piscataway, N.J.). 3′-Azido-3′-deoxythymidine 5′-triphosphate (AZTTP) was purchased from Moravek Biochemical Co. (Brea, Calif.). All other chemicals and reagents were of the highest analytical grade available.

Enzyme sources. (i) Plasmid construction. The construction and preliminary characterization of the mutations in the Tyr-Met-Asp-Asp (YMDD) regions of HIV-1 RT have been described previously (27). For the studies described here, both the mutant RT, designated M184V (methionine to valine), and the WT genes were individually amplified by PCR. The following oligonucleotides (Oligos Etc., Wilsonville, Oreg.) were used to amplify a region of the HXB2 genome from nucleotides 2095 to 3774 consisting of the RT gene in the plasmid pHXB2gpt (19): oligonucleotide 1, 5′-CTG GAT CCT TAT AGT ATT TTC CTG ATT CCA G-3′ and oligonucleotide 2, 5′-CGA CCA TGG CTC CAA TTA GCC CTA TTG AGA C-3′. The oligonucleotides were selected so that unique *Nco*I and *Bam*HI restriction sites were created 5′ and 3′, respectively, in the amplified PCR product. Following PCR, the DNA was digested with the restriction enzymes *Nco*I and *Bam*HI and the product was cloned into the expression vector pET-11d (Novagen Inc., Madison, Wis.), placing the genes under the control of the T7 promoter. As a result of the cloning into this vector, two additional amino acids, methionine and alanine, are added to the amino terminus of the expressed RT.

(ii) RT expression and purification. The above plasmids were transformed into *Escherichia coli* BL21 (DE3), which contains the T7 RNA polymerase gene under the control of the *lac* promoter. One-liter cultures were grown to an optical density at 600 nm of 0.8 before the addition of β-D-thiogalactopyranoside (0.5 mM) to induce gene expression. Cultures were centrifuged at 10,000 × *g*, and the pellets were resuspended in M-buffer (Tris-HCl [pH 7.5], 5 mM β-mercaptoethanol, 75 mM KCl, 10% glycerol) and were disrupted by sonication. After centrifugation, the soluble material was then loaded directly onto a phosphocellulose column equilibrated with 75 mM KCl in M-buffer. The bound proteins were eluted with M-buffer containing 175 mM KCl. The material was then loaded onto a DEAE-cellulose column, and the flowthrough was collected and concentrated. The purities of the enzyme preparations were evaluated to be 95% by Coomassie blue staining of sodium dodecyl sulfate-polyacrylamide gels.

Enzyme assays. Assays for RT kinetics studies contained 60 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 40 mM KCl, 2.50 mM dithiothreitol (DTT), 0.10 mg of bovine serum albumin per ml, 20 μM [^{3}H]dCTP, 2 μM poly(rI)$_n$ · oligo(dC)$_{10-15}$ (annealed in a 1:1 ratio with respect to their 5′ ends), and various concentrations of inhibitor in a final volume of 40 μl. The solution was equilibrated at 37°C, and the reaction was initiated by the addition of 0.2 μg of the WT or mutant recombinant RT. All of the assays were performed for 30 min at 37°C. DNA polymerase α and β activities were measured in 40-μl volumes by the procedures detailed by the manufacturer. All of the assays were performed for 60 min at 37°C. The reaction mixtures were then treated as described previously (23). Linear, steady-state rates were determined by linear regression analysis. The experiments were performed under conditions that led to linear reaction rates. The amount of product formed was proportional to the incubation time of the substrate intervals studied. Dixon plot analysis was used to determine inhibition constants (7).

Sequencing procedure. Sequencing procedures and acrylamide gel electrophoresis were performed as recommended in the sequencing kit. The nucleotide analog reaction mixture contained 60 mM Tris-HCl (pH 8.0), 40 mM KCl, 1 mM DTT, 2 μM dTTP, 2 μM dCTP, 2 μM dGTP, 5 mCi of [^{32}P]dATP (3,000 Ci/mmol) per ml, 0.15 pmol of single-stranded M13mp18 plus-strand DNA, 3.50 pmol of 17-base primer (universal primer −40), and various concentrations of inhibitor. The reaction was initiated by adding 0.5 μg of WT type or mutant HIV-1 RT in a total volume of 5.5 μl. Samples were incubated for 15 min at 37°C and were chased for an additional 15 min at 37°C with 0.5 μl of a solution containing the four natural deoxynucleoside triphosphates to a final concentration of 10 μM. The reactions were terminated by the addition of 4 μl of formamide stop solution, and the DNA reaction products were analyzed by using autoradiographs of wedge polyacrylamide sequencing gels.

mtDNA content analysis. Isolation, purification, culture of human bone marrow progenitor cells (CD34$^+$ cells) into granulocyte-monocyte (GM) lineages, and slot blot analysis were performed as described previously (9).

RESULTS

Effects of L-ddCTP and L-FddCTP on HIV-1 RT. L-ddCTP and L-FddCTP proved to be potent competitive inhibitors of HIV-1 RT (WT) with respect to dCTP incorporation when poly(rI)$_n$ · oligo(dC)$_{10-15}$ was used as the template primer. L-ddCTP was less inhibitory than its natural β-D analog ddCTP to WT HIV-1 RT (K_is, 2 and 1.1 μM, respectively). In contrast, the β-L and β-D enantiomers of F-ddCTP exhibited similar inhibitory effects toward HIV-1 RT (K_is, 1.35 and 1.60 μM, respectively). L-ddCTP and L-FddCTP exhibited much higher K_is with the mutant M184V HIV-1 RT (56- to 60-fold), whereas D-ddCTP and D-FddCTP showed slight elevations in their K_is (2-fold). 3TCTP, which was used as a control, exhibited a K_i of 0.95 and 88 μM toward the parental and mutant HIV-1 RTs, respectively. By using poly(rA)$_n$ · oligo(dT)$_{10}$ as a template primer, AZTTP was the most potent HIV-1 RT inhibitor toward both WT and mutant enzymes (Table 1).

Effects of L-ddCTP and L-FddCTP on human DNA polymerase α. None of the cytidine analogs demonstrated an inhibitory effect on human DNA polymerase α even at a concentration as high as 100 μM (Table 2). This enzyme exhibited a steady-state K_m of 4 μM.

Effects of L-ddCTP and L-FddCTP on human DNA polymerase β. The β-D enantiomers of ddCTP and FddCTP were

TABLE 1. Comparative inhibitory activities of L- and D-nucleoside triphosphates on WT and mutant (M184V) HIV-1 RT[a]

Inhibitor	WT RT		M184V RT		Ratio (K_i mutant/ K_i WT)
	K_i (μM)[b]	K_m/K_i[c]	K_i (μM)	K_m/K_i	
D-ddCTP	1.1 ± 0.10	6.4	2.2 ± 0.5	4.1	2
L-ddCTP	2.0 ± 0.25	3.5	120 ± 20	0.08	60
D-FddCTP	1.35 ± 0.15	5.2	3 ± 0.35	3	2
L-FddCTP	1.60 ± 0.10	4.4	90 ± 5	0.1	56
3TCTP	0.95 ± 0.08	7.4	85 ± 8	0.1	90
AZTTP[d]	0.0075 ± 0.003	120	0.025 ± 0.05	48	3

[a] Assays were performed as described in Materials and Methods. Inhibitors were analyzed by Dixon plot analysis.

[b] Each set of data represents the arithmetic mean value of the standard deviation of at least three independent experiments. Each set of data represents the arithmetic mean value of the standard deviation of at least three independent experiments.

[c] The K_m value was determined by using poly(rI)$_n$ · oligo(dC)$_{10-15}$ as the template primer and dCTP as the substrate. Under these conditions, the calculated mean K_m for dCTP was approximately 7 μM for WT RT and 9 μM for the M184V RT.

[d] The K_i of AZTTP was determined by using poly(rA)$_n$ · oligo(dT)$_{10}$ as the template primer and dTTP as the substrate. The calculated mean K_m for dTTP was approximately 0.9 μM for WT RT and 1.2 μM for the M184V RT.

found to be potent inhibitors of human DNA polymerase β, with K_i values of 0.50 and 2.50 μM, respectively (Table 2). In contrast, β-L-ddCTP and β-L-FddCTP did not exhibit any inhibitory effect up to 100 μM. AZTTP inhibited DNA polymerase β, but with a K_i of 40 μM.

Incorporation of L-ddCTP and L-FddCTP into DNA. Both L-ddCTP and L-FddCTP were analyzed for their chain-terminating activities and were compared with their D enantiomers (Fig. 1). When the WT RT was used, L-ddCTP and L-FddCTP acted as chain terminators at every 2′-deoxycytidine residue. D-ddCTP and D-FddCTP produced DNA chain terminators at similar positions, with stronger effects at the earlier 2′-deoxycytidine residues, indicating more premature DNA chain terminations (Fig. 1). 3TCTP and AZTTP, which were used as positive controls, exhibited terminations at the anticipated stops. When the mutant RT was used, L-ddCTP and L-FddC did not act as DNA chain terminators, whereas D-ddCTP and D-FddCTP exhibited DNA chain terminations similar to those found with the parental RT. 3TCTP exhibited DNA chain terminations toward the mutant RT, although this effect was less significant than that with the parental enzyme. AZTTP exhibited effects similar to those observed with the parental WT enzyme.

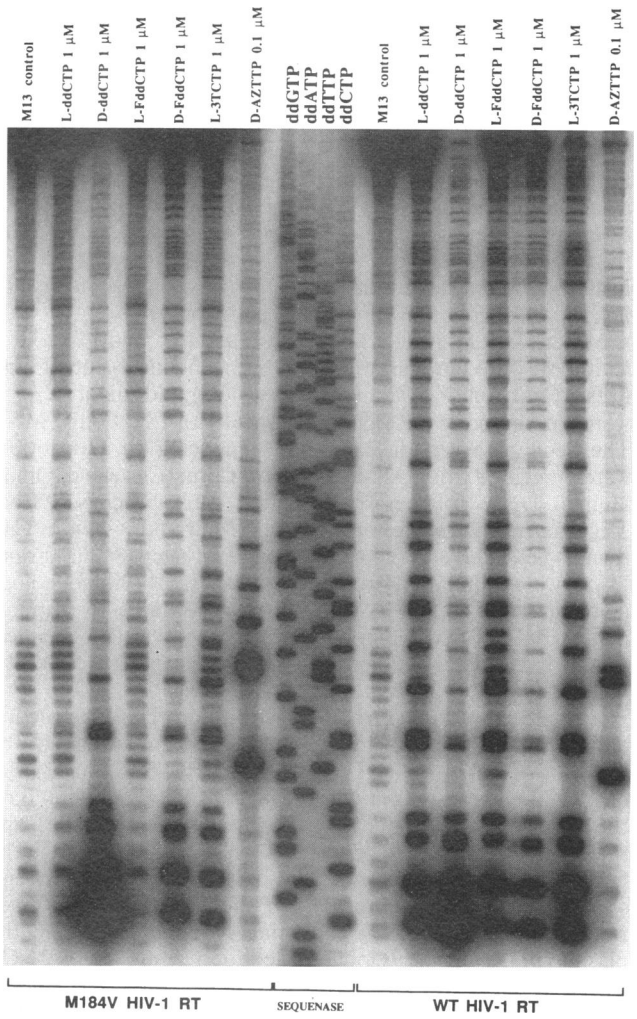

FIG. 1. Autoradiograph of chain-terminating sequencing reactions with deoxynucleoside triphosphate analogs by using WT or mutant (M184V) HIV-1 RT. Experimental conditions are described in Materials and Methods.

TABLE 2. Comparative inhibitory activities of L- and D-nucleoside triphosphates on human DNA polymerases α and β[a]

Inhibitor	DNA polymerase α		DNA polymerase β	
	K_i (μM)	K_m/K_i[b]	K_i (μM)[c]	K_m/K_i
D-ddCTP	>100	NA[d]	0.5 ± 0.3	10
L-ddCTP	>100	NA	>100	NA
D-FddCTP	>100	NA	2.5 ± 0.8	2
L-FddCTP	>100	NA	>100	NA
AZTTP	>100	NA	40 ± 5	0.05

[a] Assays were performed as described in Materials and Methods. Inhibitors were analyzed by Dixon plot analysis.

[b] The K_m value was determined by using calf thymus-activated DNA as the template primer and dCTP or dTTP as the substrate. Under these conditions, the calculated mean K_m values of human DNA polymerase α for dCTP and dTTP were approximately 4 and 1.5 μM, respectively. Human DNA polymerase β exhibited a steady-state K_m of 5 μM for dCTP and 2 μM for dTTP.

[c] Each set of data represents the arithmetic mean value of the standard deviation of at least three independent experiments. Each set of data represents the arithmetic mean value of the standard deviation of at least three independent experiments.

[d] NA, not applicable.

Effects of L-ddC and L-FddC on mtDNA content in CFU-GM liquid culture. By measuring the ratio of the mtDNA content over that of the nuclear DNA content, D-ddC strongly inhibited the mtDNA content in a dose-related manner, with 88% inhibition at 1 μM (Table 3), clearly demonstrating the preferential effect of D-ddC on mtDNA synthesis. D-FddC also inhibited the mtDNA content of cells in GM cultures, but to a lesser degree than D-ddC, with 90% inhibition at 10 μM. In contrast, L-ddC and L-FddC had no inhibitory effect on mtDNA content at concentrations (10 μM) inhibitory to cell growth (Table 3).

DISCUSSION

3TC and FTC have been shown to be the first enantiomeric natural nucleoside analogs with the unusual L configuration to be markedly more potent than their D-enantiomer counterparts against HIV-1 (5, 6, 21, 24) and HBV (8, 10) in vitro. We

TABLE 3. Effects of L- and D-cytidine analogs on mtDNA content of human CFU-GM cultures

Compound	Concn (μM)	Ratio of mtDNA/actin[a]	% Decrease in mtDNA
Control		0.82 ± 0.02	0
D-ddC	0.1	0.50 ± 0.05	39
D-ddC	1.0	0.10 ± 0.04	88
L-ddC	0.1	0.95 ± 0.06	NM[b]
L-ddC	1.0	1.07 ± 0.10	NM
L-ddC	10	1.20 ± 0.04	NM
L-FddC	0.1	0.93 ± 0.02	NM
L-FddC	1.0	1.04 ± 0.07	NM
L-FddC	10	1.24 ± 0.11	NM
D-FddC	0.1	0.84 ± 0.07	NM
D-FddC	1.0	0.52 ± 0.04	37
D-FddC	10	0.04 ± 0.02	95

[a] Each set of data represents the arithmetic mean value of the standard deviation of at least three independent experiments.
[b] NM, not measurable.

recently reported the antiviral activities of two other L-enantiomer analogs, L-ddC and its 5-fluoro-derivative L-FddC, against various strains of HIV-1 and HIV-2 in different cell lines including peripheral blood mononuclear cells and cells of the monocyte-macrophage lineage (13, 14). L-ddC and L-FddC were also shown to potently inhibit human HBV replication in vitro (22). In agreement with our data, two other groups have also recently reported the antiviral activities of L-ddC and L-FddC against HIV-1 and HBV in vitro (18, 25). L-ddC and L-FddC showed cross-resistance to 3TC- and FTC-resistant HIV-1 variants (14). We have previously reported that the mutation of the methionine at position 184 to valine was involved in 3TC and FTC resistance (23). The Met-184 residue lies in a highly conserved polymerase motif (YMDD) adjacent to the putative catalytic site of the reverse transcriptase composed of the carboxylate triad Asp-110, Asp-185, and Asp-186 (27). The mutation to valine at position 184 did not result in any decrease in viral replication, whereas mutations to serine or alanine resulted in a lower level of viral replication (11, 27). By using the homopolymer poly(rI)$_n$ · oligo(dC)$_{10-15}$ as the template primer, the 5′-triphosphates of cytidine analogs competitively inhibited parental HIV-1 RT with respect to dCTP. The D enantiomer of ddCTP demonstrated a more potent inhibition of WT HIV-1 RT than the L enantiomer, with an approximately twofold lower K_i value (Table 1). However, the replacement of the 5-position hydrogen by fluorine has led to the loss of the enantioselectivity by WT RT, since the L and D enantiomers of FddCTP equally inhibited RNA-directed DNA synthesis. These results are consistent with those reported with the L and the D enantiomers of 3TC and FTC triphosphates. Hart et al. (16) have shown that the D enantiomer of 3TC triphosphate (D-3TC) is more potent than 3TCTP in inhibiting the HIV-1 RT, whereas D-2′,3′-dideoxy-5-fluoro-3′-thiacytidine 5′-triphosphate (D-FTCTP) and L-FTCTP have been shown to inhibit HIV-1 RT equally (29). The differential in vitro antiviral activities of D-2′,3′-dideoxy-5-fluoro-3′-thiacytidine (D-FTC) and L-FTC have been suggested to result from different metabolic and catabolic pathways rather than an enantioselectivity at the RT target (24). It seems that substitution of the 5-position hydrogen by fluorine enhances the affinities of the L enantiomer analogs versus those of their D-enantiomer counterparts toward HIV-1 RT. The mutation at position 184 to valine, which is associated with resistance to 3TC and FTC, resulted in significant increases (50- to 60-fold) in K_i values for L-ddCTP and L-FddCTP, whereas the elevation in K_i values for D-ddCTP and D-FddCTP was moderate (2-fold). HIV-1 with the M184V mutation was shown to be cross-resistant to D-ddC and 2′,3′-dideoxyinosine (ddI), but the level of resistance was low (12). However, Schinazi et al. (23) was unable to demonstrate any cross-resistance with D-ddC in cell culture or using HIV-1 RT derived from virus particles. Recently, a mutation of lysine to arginine at position 65 of the HIV-1 RT that encodes HIV-1 resistance to ddC was identified (15, 31). Lys-65 is located on "fingers" subdomain of the RT crystal structure, whereas Met-184 is located on the "palm" subdomain (17).

By using a sequencing method modified from that of Sanger et al. (20), the chain-terminating activities of the L- and D-nucleoside analog inhibitors toward the WT and the mutant HIV-1 RTs were assessed. A stretch of 120 bases on the M13mp18 plus strand was used for the analysis, which allows for a better comparison of the number and the potency of incorporation of each drug (Fig. 1). L-ddCTP and L-FddCTP were demonstrated to act as potent chain-terminating agents toward WT HIV-1 RT. In contrast, L-ddCTP and L-FddCTP were not substrates for the mutant RT because no incorporation into DNA was observed (Fig. 1). It seems that 3TCTP is still the substrate for the mutant RT, although it is not inhibitory to that enzyme. These results showed that although the ribosyl portion of the substrate is not necessary for the enzyme-binding site, since HIV-1 RT accepts nucleotide analogs containing an "inverted" sugar ring (26), the methionine residue at position 184 is particularly necessary for the enzyme catalytic site for L-enantiomer analogs of nucleoside.

It is important to evaluate the effects of these L- and D-enantiomer triphosphates on human DNA polymerases to determine the selectivities of these drugs toward HIV-1 RT (4). Inhibition of these enzymes could lead to cytotoxicity, limiting the usefulness of these compounds. None of these L- or D-enantiomer analogs inhibited the human DNA polymerase α, the enzyme primarily involved in cell replication, up to a concentration of 100 μM (Table 2). The D enantiomers of ddCTP and FddCTP seemed to preferentially inhibit human DNA polymerase β, whereas the corresponding L enantiomers did not show any inhibition up to 100 μM. The inhibition of DNA polymerase β, an enzyme involved in the DNA repair processes, could lead to an accumulation of damaged DNA, and could thereby be involved in some cytotoxic events induced by these compounds. D-ddC, along with AZT and ddI, are the only drugs approved for the treatment of HIV infection. The dose-limiting toxicity of ddC is severe neuropathy (30), which has been suggested to be the result of an inhibition of mtDNA synthesis subsequent to inhibition of mtDNA polymerase γ by D-ddCTP (2, 3). In contrast, use of L-ddC and L-FddC did not result in any decrease in mtDNA content in human bone marrow cells after 14 days of culture when they were used up to a concentration inhibitory to cell growth (10 μM), whereas the D enantiomers D-ddC and D-FddC inhibited mtDNA synthesis, by 90% at concentrations of 1 and 10 μM, respectively (Table 3). Consistent with these data, Lin et al. (18) have also recently suggested that L-ddC and L-FddC did not demonstrate any effect on mtDNA synthesis in CEM cells up to 100 μM. The lack of inhibition of mtDNA content by the L enantiomers of dideoxycytidine and its 5-fluoro derivative may reflect a limited affinity of these compounds toward DNA polymerase γ or the lack of transport of their triphosphate derivatives from the cytoplasm to the mitochondria, as previously suggested for other derivatives (1, 3).

The potent inhibition of L-ddCTP and L-FddCTP toward the viral RT concomitant to their lack of effects on the host cell DNA polymerases suggests that the use of L-cytidine analogs,

nd in particular L-FddC, may be warranted for further evaluation of their use in the treatment of HIV infection.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants AI-33239 (J.-P.S.), AI-25899 (R.F.S.), and AI-34749 (C.D.M.); by the U.S. Department of Veterans Affairs; by a grant from CNRS, INSERM, Agence Nationale de Recherche sur le SIDA, Paris, France (G.G. and J.-L.I.); and the UAB AIDS Center, Molecular Biology Core Facility (P30AI27767). J.-P.S. is the recipient of a Faculty Research Award from the American Cancer Society.

We thank Terri Hicks for preparing and editing the manuscript.

REFERENCES

1. **Chang, C. N., V. Skalski, J. H. Zhou, and Y.-C. Cheng.** 1992. Biochemical pharmacology of (+)- and (−)-2′,3′-dideoxy-3′-thiacytidine as anti-hepatitis B virus agents. J. Biol. Chem. **267:**22414–22420.
2. **Chen, C. H., and Y.-C. Cheng.** 1989. Delayed cytotoxicity and selective loss of mitochondrial DNA in cells treated with the anti-human immunodeficiency virus compound 2′,3′-dideoxycytidine. J. Biol. Chem. **264:**11934–11937.
3. **Chen, C. H., and Y.-C. Cheng.** 1992. The role of cytoplasmic deoxycytidine kinase in the mitochondrial effects of the anti-human immunodeficiency virus compound, 2′,3′-dideoxycytidine. J. Biol. Chem. **267:**2856–2859.
4. **Cheng, Y.-C., C. H. Chen, M. Vazquez-Padua, and M. C. Starnes.** 1990. DNA polymerases versus HIV reverse transcriptase in AIDS therapy. Ann. N.Y. Acad. Sci. **616:**217–223.
5. **Coates, J. A. V., N. Cammack, H. J. Jenkinson, A. J. Jowett, M. I. Jowett, B. A. Pearson, C. R. Penn, P. L. Rouse, K. C. Viner, and J. M. Cameron.** 1992. (−)-2′-Deoxy-3′-thiacytidine is a potent, highly selective inhibitor of human immunodeficiency virus type 1 and type 2 replication in vitro. Antimicrob. Agents Chemother. **36:**733–739.
6. **Coates, J. A. V., N. Cammack, H. J. Jenkinson, I. M. Mutton, B. A. Pearson, R. Storer, J. M. Cameron, and C. R. Penn.** 1992. The separated enantiomers of 2′-deoxy-3′-thiacytidine (BCH 189) both inhibit human immunodeficiency virus replication in vitro. Antimicrob. Agents Chemother. **36:**202–205.
7. **Dixon, M.** 1953. The determination of enzyme inhibitory constants. Biochem. J. **55:**170–171.
8. **Doong, S.-L., C.-H. Tsai, R. F. Schinazi, D. C. Liotta, and Y.-C. Cheng.** 1991. Inhibition of the replication of hepatitis B virus in vitro by 2′,3′-dideoxy-3′-thiacytidine and related analogues. Proc. Natl. Acad. Sci. USA **88:**8495–8499.
9. **Faraj, A., D. A. Fowler, E. G. Bridges, and J.-P. Sommadossi.** 1994. Effects of 2′,3′-dideoxynucleosides on the proliferation and differentiation of human pluripotent progenitors in liquid culture and their effects on mitochondrial DNA synthesis. Antimicrob. Agents Chemother. **38:**924–930.
10. **Furman, P. A., M. Davis, D. C. Liotta, M. Paff, L. W. Frick, D. J. Nelson, R. E. Dornsife, J.-A. Wurter, L. J. Wilson, J. A. Fyfe, J. V. Tuttle, L. Condrey, D. R. Averett, R. F. Schinazi, and G. R. Painter.** 1993. The anti-hepatitis B virus activities, cytotoxicities, and anabolic profiles of the (−) and (+) enantiomers of *cis*-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine. Antimicrob. Agents Chemother. **36:**2423–2431.
11. **Gao, Q., Z. Gu, J. Hiscott, G. Dionne, and M. A. Wainberg.** 1993. Generation of drug-resistant variants of human immunodeficiency virus type 1 by in vitro passage in increasing concentrations of 2′,3′-dideoxycytidine and 2′,3′-dideoxy-3′-thiacytidine. Antimicrob. Agents Chemother. **37:**130–133.
12. **Gao, Q., Z. Gu, M. A. Parniak, J. Cameron, N. Cammack, C. Boucher, and M. A. Wainberg.** 1993. The same mutation that encodes low-level human immunodeficiency virus type 1 resistance to 2′,3′-dideoxyinosine, and 2′,3′-dideoxycytidine confers high-level resistance to the (−) enantiomer of 2′,3′-dideoxy-3′-thiacytidine. Antimicrob. Agents Chemother. **37:**1390–1392.
13. **Gosselin, G., C. Mathé, M.-C. Bergogne, A. M. Aubertin, A. Kirn, R. F. Schinazi, J.-P. Sommadossi, and J.-L. Imbach.** 1994. Enantiomeric 2′,3′-dideoxycytidine derivatives are potent human immunodeficiency virus inhibitors in cell cultures. C. R. Acad. Sci. Ser. III **317:**85–89.
14. **Gosselin, G., R. F. Schinazi, J.-P. Sommadossi, C. Mathé, M.-C. Bergogne, A. M. Aubertin, A. Kirn, and J.-L. Imbach.** Anti-human immunodeficiency virus activities of the β-L-enantiomer of 2′,3′-dideoxycytidine and its 5-fluoro derivative. Antimicrob. Agents Chemother. **38:**1292–1297.
15. **Gu, Z., Q. Gao, H. Fang, H. Salomon, M. A. Parniak, E. Goldberg, J. Cameron, and M. A. Wainberg.** 1994. Identification of a mutation at codon 65 in the IKKK motif of reverse transcriptase that encodes human immunodeficiency virus resistance to 2′,3′-dideoxycytidine and 2′,3′-dideoxy-3′-thiacytidine. Antimicrob. Agents Chemother. **38:**275–281.
16. **Hart, G. J., D. C. Orr, R. Penn, H. T. Figueiredo, N. M. Gray, E. Boehme, and J. Cameron.** 1992. Effects of (−)-2′-deoxy-3′-thiacytidine (3TC) 5′-triphosphate on human immunodeficiency virus reverse transcriptase and mammalian DNA polymerases alpha, beta, and gamma. Antimicrob. Agents Chemother. **36:**1688–1694.
17. **Kohlstaedt, L. A., J. Wang, J. M. Friedman, P. A. Rice, and T. A. Steitz.** 1992. Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. Science **256:**1783–1790.
18. **Lin, T.-S., M.-Z. Luo, M.-C. Liu, S. B. Pai, G. E. Dutschman, and Y.-C. Cheng.** 1994. Antiviral activity of 2′,3′-dideoxy-β-L-fluorocytidine (β-L-FddC) and 2′,3′-dideoxy-β-L-cytidine (β-L-ddC) against hepatitis B virus and human immunodeficiency virus type 1 in vitro. Biochem. Pharmacol. **47:**171–174.
19. **Ratner, L., A. Fisher, L. Linda, J. Agodzinski, H. Mitsuya, R. S. Liou, R. C. Gallo, and F. Wong-Staal.** 1987. Complete nucleotide sequences of functional clones of the AIDS virus. AIDS Res. Hum. Retroviruses **3:**57–69.
20. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
21. **Schinazi, R. F., C. K. Chu, A. Peck, A. McMillan, R. Mathis, D. Cannon, L.-S. Jeong, J. W. Beach, W.-B. Choi, S. Yeola, and D. C. Liotta.** 1992. Activity of the four optical isomers of 2′,3′-dideoxy-3′-thiacytidine (BCH-189) against HIV-1 in human lymphocytes. Antimicrob. Agents Chemother. **36:**672–676.
22. **Schinazi, R. F., G. Gosselin, A. Faraj, B. R. Korba, D. C. Liotta, C. K. Chu, C. Mathé, J.-L. Imbach, and J.-P. Sommadossi.** Pure nucleoside enantiomers of β-2′,3′-dideoxycytidine analogs are selective inhibitors of hepatitis B virus and human immunodeficiency virus in vitro. Antimicrob. Agents Chemother., in press.
23. **Schinazi, R. F., R. M. Llold, Jr., M.-H. Nguyen, D. L. Cannon, A. Mcmillan, N. Ilksoy, C. K. Chu, D. C. Liotta, H. Z. Bazmi, and J. W. Mellors.** 1993. Characterization of human immunodeficiency viruses resistant to oxathiolane-cytosine nucleosides. Antimicrob. Agents Chemother. **37:**875–881.
24. **Schinazi, R. F., A. McMillan, D. Cannon, R. Mathis, R. M. Lyod, A. Peck, J.-P. Sommadossi, M. St. Clair, J. Wilson, P. A. Furman, G. Painter, W.-B. Choi, and D. C. Liotta.** 1992. Selective inhibition of human immunodeficiency viruses by racemates and enantiomers of *cis*-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine. Antimicrob. Agents Chemother. **36:**2423–2431.
25. **Van Draanen, N. A., M. Tisdale, N. R. Parry, R. Jansen, R. E. Dornsife, J. V. Tuttle, D. R. Averett, and G. W. Koszalka.** 1994. Influence of stereochemistry on antiviral activities and resistance profiles of dideoxycytidine nucleosides. Antimicrob. Agents Chemother. **38:**868–871.
26. **Van Draanen, N. A., S. C. Tucker, F. L. Boyd, B. W. Trotter, and J. E. Reardon.** 1992. β-L-Thymidine 5′-triphosphate analogs as DNA polymerase substrate. J. Biol. Chem. **267:**25019–25024.
27. **Wakefield, J. K., S. A. Jablonski, and C. D. Morrow.** 1992. In vitro enzymatic activity of human immunodeficiency virus type 1 reverse transcriptase mutants in the highly conserved YMDD amino acid motif correlates with the infectious potential of the proviral genome. J. Virol. **66:**6806–6812.
28. **White, E. L., W. B. Parker, L. J. Macy, S. C. Shaddix, G. McCaleb, J. A. I. Secrist, R. Vince, and W. M. Shannon.** 1989. Comparison of the effect of carbovir, AZT, and dideoxynucleoside triphosphates on the activity of human immunodeficiency virus reverse transcriptase and selected human polymerases. Biochem. Biophys. Res. Commun. **161:**393–398.

29. **Wilson, J. E., J. L. Martin, K. Borroto-Esoda, S. Hopkins, G. Painter, D. C. Liotta, and P. A. Furman.** 1993. The 5′-triphosphates of the (−) and (+) enantiomers of *cis*-5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine equally inhibit human immunodeficiency virus type 1 reverse transcriptase. Antimicrob. Agents Chemother. **37:**1720–1722.

30. **Yarchoan, R., C. F. Perno, R. V. Thomas, R. W. Klecker, J.-P. Allain, R. J. Wills, N. McAtee, M. A. Fischl, R. Dubinsky, M. C. McNealy, H. Mitsuya, J. M. Pluda, T. J. Lawley, M. Leuther, B. Safai, J. M. Collins, C. E. Myers, S. Broder.** 1988. Phase I studies of 2′,3′-dideoxycytidine in severe human immunodeficiency virus infection as a single agent and alternating with zidovudine (AZT). Lancet **i:**76–81.

31. **Zhang, D., A. M. Caliendo, J. J. Eron, K. M. Devore, J. C. Kaplan, M. S. Hirsh, and R. T. D'aquila.** 1994. Resistance to 2′,3′-dideoxycytidine conferred by a mutation in codon 65 of the human immunodeficiency virus type 1 reverse transcriptase. Antimicrob. Agents Chemother. **38:**282–287.