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Selective Inhibition of Human Immunodeficiency Viruses by Racemates and Enantiomers of *cis*-5-Fluoro-1-[2-(Hydroxymethyl)-1,3-Oxathiolan-5-yl]Cytosine

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2′,3′-Dideoxy-5-fluoro-3′-thiacytidine (FTC) has been shown to be a potent and selective compound against human immunodeficiency virus type 1 in acutely infected primary human lymphocytes. FTC is also active against human immunodeficiency virus type 2, simian immunodeficiency virus, and feline immunodeficiency virus in various cell culture systems, including human monocytes. The antiviral activity can be prevented by 2′-deoxycytidine, but not by other natural nucleosides, suggesting that FTC must be phosphorylated to be active and 2′-deoxycytidine kinase is responsible for the phosphorylation. By using chiral columns or enzymatic techniques, the two enantiomers of FTC were separated. The (−)-β-enantiomer of FTC was about 20-fold more potent than the (+)-β-enantiomer against human immunodeficiency virus type 1 in peripheral blood mononuclear cells and was also effective in thymidine kinase-deficient CEM cells. Racemic FTC and its enantiomers were nontoxic to human lymphocytes and other cell lines at concentrations of up to 100 μM. Studies with human bone marrow cells indicated that racemic FTC and its (−)-enantiomer had a median inhibitory concentration of >30 μM. The (+)-enantiomer was significantly more toxic than the (−)-enantiomer to myeloid progenitor cells. The susceptibilities to FTC of pretherapy isolates in comparison with those of posttherapy 3′-azido-3′-deoxythymidine-resistant viruses in human lymphocytes were not substantially different. Similar results were obtained with well-defined 2′,3′-dideoxyinosine- and nevirapine-resistant viruses. (−)-FTC-5′-triphosphate competitively inhibited human immunodeficiency virus type 1 reverse transcriptase, with an inhibition constant of 2.9 μM, when a poly(I)$_n$ · oligo(dC)$_{19-24}$ template primer was used. A two- to threefold decreased affinity was noted for the (+)-enantiomer. By using sequencing analysis, racemic FTC-5′-triphosphate was shown to be a potent DNA chain terminator when human immunodeficiency virus reverse transcriptase was used. These results suggest that further development of the (−)-β-enantiomer of FTC is warranted as an antiviral agent for infections caused by human immunodeficiency viruses.

The introduction of 3′-azido-3′-deoxythymidine (AZT) as the drug of choice for the treatment of infections caused by human immunodeficiency virus type 1 (HIV-1) has generated interest in the development of additional nucleosides as well as nonnucleoside antiviral agents (40). The development of antiviral agents with improved therapeutic indices and pharmacological profiles is highly desirable. Our objective was to synthesize compounds that are sufficiently different from natural nucleosides so that they would be recognized by viral enzymes but not by certain cellular enzymes. Structure-activity relationship studies with pyrimidine nucleosides modified at the 5 position indicated that toxicity can be markedly reduced by having this position modified with a small group such as a halogen or an alkyl group not larger than propyl without markedly compromising their activities. However, certain pyrimidine nucleosides unsubstituted at the 5 position are also effective and maintain selectivity, at least in culture (19). The recent development of efficient chemical methods for the stereoselective syntheses of nucleosides with an oxathiolane ring (4, 16) and the discovery by Belleau et al. (3) of the antiviral activity of racemic 2′,3′-dideoxy-3′-thiacytidine (BCH-189) prompted us to examine the structure activity of analogs of this compound, especially modifications of pyrimidine nucleosides at the 5 position. Substitution at the 5 position may also reduce the affinity of cytidine analogs for cytidine deaminase (34). Among this series, 2′,3′-dideoxy-5-fluoro-3′-thiacytidine (FTC) was found to be very potent and selective against HIV-1 and HIV-2. Since these oxathiolane nucleosides have two diastereoisomers and each diastereoisomer has two enantiomers, the activities and toxicities of the enantiomers of FTC were explored. This led to the finding that the (−)-β-isomer of FTC [(−)-FTC] is, in general, more potent than the (+)-β-enantiomer [(+)-FTC] (Fig. 1). The complete chemical name for (−)-FTC, on the basis of the crystal structure determined by P. Van Roey (Albany, N.Y.), is (2′-*R*,5′-*S*)-(−)-1-[2-hydroxymethyl)oxathiolan-5-yl]-5-fluorocytosine. These unexpected properties are similar to those recently reported by British and Canadian groups and independently

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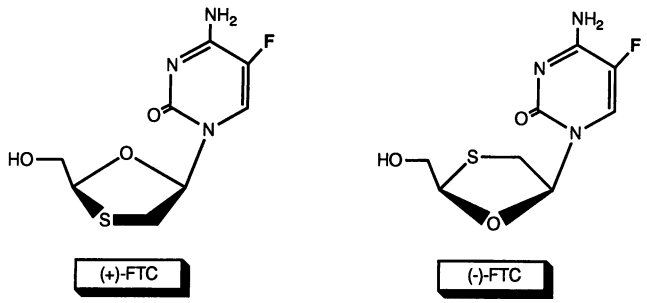

FIG. 1. Structures of the two antiviral enantiomers of FTC.

by our group with the (−)-enantiomer of BCH-189 [(−)-BCH-189; also known as 3TC] (6, 7, 35, 45). In this report we describe the antiviral properties of FTC and its enantiomers relative to those of AZT, BCH-189, and the antiviral enantiomers of BCH-189. The mechanisms of action of FTC and its enantiomers were explored with HIV-1 reverse transcriptase (RT).

MATERIALS AND METHODS

Compounds. All the 3′-thiapyrimidine nucleosides were synthesized in our laboratories and were characterized on the basis of their physical (combustion analysis, melting points, high-pressure liquid chromatographic [HPLC] elution time) and spectroscopic (^1H and ^{13}C nuclear magnetic resonance) properties. The detailed synthesis of these compounds as well as their (+)- and (−)-enantiomers, which were obtained by using lipases and cytidine deaminase, are reported elsewhere (4, 16, 20, 21). The optical rotations $[\alpha]_D$ for the (+)- and (−)-enantiomers of FTC, as determined in absolute ethanol (c = 0.88) on a Perkin-Elmer 241MC polarimeter, were +113.4° and −120.5°, respectively. Racemic and enantiomeric FTC-5′-triphosphates (FTC-TPs) were synthesized by standard phosphorylating methods (12, 48). AZT, which was used as a control, was synthesized by the method of Lin et al. (19). AZT-5′-triphosphate (AZT-TP) was synthesized in our laboratory as described previously (12). The purity of all the nucleosides was >99%, as determined by HPLC. Other nucleosides and nucleotides, such as 2′,3′-dideoxycytidine and its 5′-triphosphate, were obtained from U.S. Biochemical Corp., Cleveland, Ohio. The TIBO analog R82150 was obtained from K. Parker (Brown University, Providence, R.I.).

Cells, virus strains, and cell culture assays. Human peripheral blood mononuclear (PBM) cells from healthy donors who were seronegative for HIV-1 and hepatitis B virus were isolated by a single-step Ficoll-Hypaque discontinuous gradient centrifugation (Sigma, St. Louis, Mo.) and were propagated as described previously (29, 31). A prototype strain of HIV-1 (LAV) obtained from the Centers for Disease Control, Atlanta, Ga., was used as the standard virus for most of the antiviral assays. Details on the infection of cells and assessment of antiviral effects have been reported previously (31). A multiplicity of infection of 0.1, as determined by a limiting dilution method in PBM cells, was selected for the assays. This represents about 50 50% tissue culture infective doses per 25-cm^2 flask per 5×10^6 cells per 10 ml. Furthermore, peak RT levels occur within 6 to 7 days when this multiplicity of infection is used. Antiviral assays conducted at Burroughs Wellcome Co. were performed in PBM cells as described previously (46). AZT-resistant and -susceptible virus strains 9F (G910-6) and 10 (H112-2) were obtained from D. Richman (Veterans Affairs Medical Center, San Diego, Calif.) through the AIDS Research and Reference Program, National Institutes of Health, and were propagated in PBM cells as described previously (31). Similarly, the nevirapine (Bl-RG587)-resistant HIV-1 strain derived from strain LAV obtained from D. Richman was used (27). CEM (CEM-CCRF) cells are a T-lymphoblastoid cell line obtained from the American Type Culture Collection, Rockville, Md. These cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) as described previously (31, 32). Thymidine kinase (TK)-deficient (TK^-) CEM cells were prepared in our laboratory as described previously (35). The viruses obtained from the PBM cell supernatant were titrated and stored in aliquots at −70°C until use. A 50% tissue culture infective dose of about 100 was used to infect the CEM cells, and virus was harvested 6 and 14 days after infection. For most of the assays, AZT as well as racemic BCH-189 and its enantiomers were included for comparison.

Low-passage HIV-1 clinical isolates 2:DR1 and 2:DR2 were obtained from L. Resnick (Mt. Sinai Medical Center, Miami, Fla.). Subsequently, it was determined that isolate 2:DR1 is AZT resistant and contains the Thr-215 to Tyr mutation (see Table 1). This isolate was cultured from a patient with AIDS who had received AZT for over 1 year and whose $CD4^+$ counts were less than 200/μl of blood. Isolate J6 was obtained from an asymptomatic HIV-1-positive person who had never received AZT. The AZT-resistant M6 isolate was obtained from a patient with AIDS who had received long-term AZT treatment and contains the Met-41 to Leu and Thr-215 to Tyr mutations (46). The 2′,3′-dideoxyinosine (DDI)-resistant M48 isolate was obtained from the same patient 11 months after the patient's treatment was switched from AZT to DDI. This virus contained the Thr-215 to Tyr and Leu-74 to Val mutations (46). The susceptibilities of clinical isolates to FTC and its enantiomers were determined in PBM cells as described previously (46) or by using the standardized assay described above. The methodology used for analyzing HIV_{IIIB} in MT-4 cells has also been described previously (1, 10). The assays in human monocyte macrophages were performed by using the HIV_{IIIB} strain as described previously (10). HIV-2 strains ROD2 and Zy were originally obtained from Luc Montagnier and Daniel Zagury, respectively. Assays with these viruses were performed as described above for HIV-1.

Studies with simian immunodeficiency virus were performed in AA-2 and C-8166 cells by using strain SIV251, as described previously (47). Studies with feline immunodeficiency virus (strain petaluma) and feline leukemia virus-feline AIDS (FAIDS) (clone 61E) were performed in Crandell feline kidney (CrFK) cells as described previously (17, 25). Multiplicities of infection of 0.01 and 0.5 were used for the feline immunodeficiency virus and feline leukemia virus studies, respectively. Virus yield was determined by measuring the p26 or p27 *gag* proteins by an antigen capture enzyme immunoassay.

Delayed-treatment experiments were performed to determine whether the activities of FTC and the other antiviral agents were reduced if the drug was added at different times after infection. Infected PBM cells were exposed to the drugs on days 0, 1, 2, and 3 after infection. The virus inocula, the concentrations of the compounds used, and the harvesting and quantitation of virus have been described previously (32).

Cytotoxicity assays in lymphocytes. The compounds were evaluated for their potential toxic effects in uninfected phytohemagglutinin-stimulated human PBM cells and also in CEM and Vero (African Green monkey kidney) cells. The CEM cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). The PBM and CEM cells were cultured with and without drug for 6 days, after which time aliquots were counted for cell proliferation and viability (31). Only the effects on cell proliferation are reported since these correlated well with cell viability. The toxicities of the compounds in Vero cells were assessed after 3 days of treatment with a hemacytometer, as described previously (31). Cytotoxicity in MT-4 cells was assessed by the propidium iodide incorporation method as described previously (1).

Assay of CFU of granulocyte macrophages (CFU-GM) or burst-forming units erythroid (BFU-E) for drug cytotoxicity studies. Human bone marrow cells were collected by aspiration from the posterior iliac crest of normal healthy volunteers and treated with heparin, and the mononuclear cell population was separated by Ficoll-Hypaque gradient centrifugation as described previously (42). Cells were washed twice in Hanks balanced salt solution and counted with a hemacytometer, and their viabilities were >98% as assessed by trypan blue exclusion. The culture assays were performed by using a bilayer soft agar or methylcellulose method as described previously (42). After incubation for 14 days at 37°C in a humidified atmosphere of 5% CO_2 in air, colonies (≥50 cells) were counted with an inverted microscope.

Biochemical assays. [^{3}H]dCTP (82.3 Ci/mmol) was purchased from New England Nuclear Corp., Boston, Mass. 2,3′-Dideoxycytidine-5′-triphosphate (ddCTP) was used as a positive control. ATP (magnesium salt), natural 2′-deoxynucleoside triphosphates, calf thymus DNA, and DNase I were obtained from Sigma Chemical Co. The standard template-primers $(rI)_n \cdot (dC)_{12-18}$ and $(rI)_n \cdot (dC)_{19-24}$ used for RT assays were prepared in our laboratories by using reagents purchased from Pharmacia Fine Chemicals, Piscataway, N.J. The recombinant p66/51 heterodimer was prepared from the HXB2 clone (14). The L74V/T215Y RT was derived by site-directed mutagenesis of an RT-containing plasmid obtained from the infectious HXB2 clone. Both wild-type and mutant RTs were purified by immunoaffinity chromatography (22). For kinetic assays with the $(rI)_n \cdot (dC)_{19-24}$ template primer, the assay conditions used are described briefly. Template and primer were annealed in a 1:1 ratio with respect to their 5′ ends. Assays for kinetic studies contained 50 mM Tris (pH 7.7), 2 mM $MgCl_2$, 0.025% Triton X-100, 20 μM [^{3}H]dCTP, 2 μM $(rI)_n \cdot (dC)_{19-24}$, and various concentrations of inhibitor. The solution was equilibrated at 37°C, and the reaction was initiated with enzyme so that the final concentration was 2 to 5 nM. Six aliquots were removed over the course of the reaction, spotted on DE81 paper, washed in 125 mM Na_2HPO_4, dried, and counted. Linear, steady-state rates were determined by linear regression analysis. Dixon plot analysis for a competitive inhibitor was used to determine inhibition constants (8).

The p66/51 heterodimer RT derived from the HB10 clone was obtained from BioTechnology General, Inc., Rehovot, Israel. This material was used for the sequencing studies (see below) and the kinetic assays performed with the $(rI)_n \cdot (dC)_{12-18}$ template primer. The conditions for the kinetic assays and the methodologies used have been described previously (12, 34). Protein was determined by the method of Bradford in a microassay by using the Bio-Rad Protein Assay dye reagent concentrate obtained from Bio-Rad Laboratories, Rockville Center, N.Y. The experiments were performed in a range in which the response was linear with respect to the substrate concentration. The amount of product formed was proportional to the incubation time in the substrate intervals studied. Apparent inhibition constants were obtained from a Dixon plot (8).

HPLC assays. A Chiralpak AS 10-μm, 25-cm by 4.6-mm column (catalog no. 7406-00; J. T. Baker Inc., Phillipsburg, N.J.) was used for the separation of the enantiomers. The mobile phase was isocratic HPLC-grade isopropyl alcohol (Fisher Scientific, Pittsburgh, Pa.). The flow rate was 0.8 ml/min. The eluent was monitored by UV detection at 270 nm. The retention times for (−)-FTC, and (+)-FTC were 5.9 and 9.5 min, respectively. The (−)- and (+)-enantiomers were also resolved from racemic FTC by this method. Fractions containing each of the pure enantiomers were pooled, frozen, and then lyophilized. The compounds were characterized by UV spectroscopy and by their retention times on HPLC compared with those of the synthetic enantiomers. The concentrations of the compounds were determined by UV spectroscopy. Stock solutions of known concentrations were prepared in water for biological evaluation.

Sequencing procedure. The dideoxy-DNA chain termination sequencing procedure of Sanger et al. (28) was used to assess DNA chain termination. [^{32}P]dATP was obtained from Amersham Corp. (Arlington Heights, Ill.). The template was the M13mp18(+) strand DNA obtained from Pharmacia LKB Biotechnology (Piscataway, N.J.). All other reagents for sequencing were included in the Sequenase kit no. 70700 (U.S. Biochemical Corp.). Sequencing procedures and acrylamide gel electrophoresis were performed as recommended in the kits. Specifically, the nucleotide analog reactions contained various concentrations of compound, from 0.01 to 1.0 μM, 0.18 μM TTP, 0.18 μM dCTP, 0.18 μM dGTP, 10 mCi/ml [^{32}P]dATP (specific activity, 1,000 to 1,500 Ci/mmol), 10 mM $MgCl_2$, 0.5 pmol of single-stranded M13mp18(+) template, 20.0 pmol of 17-base primer, and 4.2 U of recombinant p66/51 RT obtained from BioTechnology General, Inc. The specific activity of this enzyme was 5,600 U/mg of protein, where 1 U of RT is defined as the amount required to incorporate 1 nmol of [^{32}P]TTP, following 10 min of polymerization, per mg of protein at 37°C. For the control (without drug or dideoxynucleoside triphosphates [ddNTPs]), the final concentration of deoxynucleoside triphosphate (dNTP) was 0.18 μM. The control ddNTP sequences were determined by using the Sequenase enzyme provided in the kit and included $MnCl_2$ (3.65 mM) in order to enhance short chain reactions. The concentrations of ddNTP and other dNTPs used for the Sequenase enzyme were 3.33 and 33.3 μM, respectively. The control lane without ddNTPs or AZT-TP contained RT, template, and primer at the same concentrations as those in the experimental lanes and the dNTPs at a final concentration of 0.18 μM. The intensity of the bands was quantitated using a Molecular Dynamics ImageQuant.

Data analyses. The median effective concentration (EC_{50}) and the median inhibitory concentration (IC_{50}) were derived from the computer-generated median effect plot of the dose-effect data as described previously (30). By using the standard error of the slope of the best-fitting line, the minimum and maximum EC_{50}s within the 95% confidence range were calculated. A lack of overlap between the ranges derived for the virus-resistant and virus-susceptible conditions would

TABLE 1. Antiretroviral activities and cytotoxicities of AZT, BCH-189, and FTC in different cell systems

Activity (virus) and cell system	Mean EC_{50}[a] or IC_{50} (μM)						
	AZT	BCH-189			FTC		
		(±)	(−)	(+)	(±)	(−)	(+)
Antiviral (HIV-1)							
PBM (LAV-1)	0.004	0.06[b]	0.002[b]	0.20[b]	0.03	0.008	0.84
CEM (LAV-1)	0.005	0.07[b]	0.07[b]	0.10[b]	0.08	0.009	1.4
	0.1[c]	0.45[c]	>100[c]	4.4[c]	0.12[c]	1.01[c]	3.9[c]
CEM-TK$^-$ (LAV-1)	>100	0.09[b]	0.09[b]	0.08[b]	0.02	0.03	0.38
	>100[c]	0.48[c]	>100[c]	4.6[c]	0.39[c]	0.49[c]	28.5[c]
PBM (HIV_{IIIB})	0.04	0.8	0.07		0.69	0.01	
CEM (HIV_{IIIB})	0.03		0.3		0.9	0.1	
MT-4 (HIV_{IIIB})	0.06	7.0	3.2		2.1	0.5	
Human monocytes (HIV_{IIIB})	0.06	3.1	0.69		0.1	0.01	
PBM (J6)	0.05		0.01			0.002	
PBM (2:DR2)	0.003					0.002	0.10
HT4-6C (LAV)						0.02	
Antiviral (HIV-2)							
PBM (ROD2)	0.004	0.001	0.02	0.05	0.004	0.0007	0.03
MT-4 (Zy)	0.04	15.0	9.8		8.1	1.5	
CEM (Zy)	0.03		0.3			0.1	
Antiviral (HIV-1 resistant)							
PBM (G910; AZT resistant)[d]	0.33[e]	0.24[e]			0.08[e]	0.05[e]	2.1[e]
PBM (H112-2; AZT susceptible)[d]	0.005	0.04			0.04	0.006	0.99
PBM (M6; AZT resistant)	13.9		0.01			0.002	
PBM (2:DR1; AZT resistant)	2.0					0.08	7.5
PBM (M48; DDI resistant)	0.73		0.05			0.01	
PBM (BI-RG587 resistant)[f]	0.004	0.0008	0.007	0.10	0.008	0.0001	0.10
HT4-6C (BI-RG587 resistant)[f]						0.02	
Cytotoxicity (uninfected)							
PBM[g]	>100	>100[b]	>100[b]	>100[b]	>100	>100	>100
CEM[h]	14.3[a]	52.6[b]	>100[b]	2.7[b]	>100	>100	>100
Vero[i]	28.0[b]	>100[b]	>100[b]	>100[b]	>100	>100	>100
MT-4	20.0	>100	>100	>100	>100	>100	>100

[a] Variance from the mean was ±8 to 50%. Values are means of duplicate or triplicate assays. The correlation coefficient for the data was ≥0.94.
[b] Taken from reference 35.
[c] Measured on day 14.
[d] These are isolates obtained from the same patient before and after treatment with AZT.
[e] Significantly different from AZT-susceptible virus ($P < 0.05$).
[f] This virus was resistant to BI-RG587 (EC_{50}, 3.6 μM in HT4-6C cells) and cross-resistant to TIBO/R82150 (EC_{50}, 1.7 μM in human PBM cells).
[g] PBM cells were counted by the trypan blue exclusion method after drug exposure for 6 days. Untreated cultures had about 4×10^5 cells per ml.
[h] CEM cells were counted after drug exposure for 6 days. Untreated cultures had about 2×10^5 cells per ml.
[i] Vero cells were counted after drug exposure for 4 days. Untreated cultures had about 3×10^5 cells per ml.

indicate a difference between these values at a $P < 0.05$ level.

RESULTS

Antiviral and cytotoxicity assays. (−)-FTC and (±)-FTC were potent inhibitors of HIV-1 (strain LAV) at submicromolar concentrations (Table 1). The (+)-enantiomer was about 28-fold less potent than (±)-FTC (Table 1). The compounds listed in their order of potency were (−)-FTC ≥ (±)-FTC > (+)-FTC. The same order of potency was found for BCH-189 and its enantiomers (Table 1). In CEM cells infected with HIV-1 (strain LAV), the potency difference between the (+)- and (−)-enantiomers of FTC was approximately 156-fold. This potency difference was much less pronounced for the BCH-189 enantiomers when virus yield was measured on day 6. Interestingly, the antiviral activity of (−)-BCH-189 was completely abolished when virus was harvested on day 14 (EC_{50}, >100 μM). In contrast, under the same conditions, the potency of (−)-FTC decreased by a factor of approximately 10 (Table 1). Mean EC_{50}s were generally 1 order of magnitude greater for HIV-1 (strain HIV_{IIIB}) than those found for the LAV strain in PBM and CEM cells. In CEM-TK$^-$ cells, the (−)- and (+)-enantiomers of FTC had EC_{50}s similar to those found in normal CEM cells. However, AZT, which is known to be phosphorylated by cellular TK, was active in CEM cells but not in CEM-TK$^-$ cells.

The anti-HIV-1 activity and lack of toxicity of FTC and its enantiomers were also confirmed in different cells, including MT-4 cells and human monocyte acutely infected with HIV-1 (Table 1). As demonstrated in human PBM cells, (+)-FTC was less potent than (−)-FTC in monocytes. At 1 μM, racemic FTC was highly effective (>90% inhibition) in PBM cells even when the drug was added 2 days after virus infection (data not shown). When the concentration was reduced to 0.1 μM, this compound was effective only when it was added 24 h after infection.

To confirm that the synthetic enantiomers were associated with the correct isomer, racemic FTC was chromatographed

TABLE 2. Effect of exogenous 2'-deoxycytidine on the antiviral activity of (−)-FTC in human PBM cells infected with HIV-1 (strain LAV)[a]

(−)-FTC concn (μM)	2'-Deoxycytidine concn (μM)	% Inhibition
0	100.0	25.8
0.1	0	98.7
0.1	1.0	92.4
0.1	10.0	61.1
0.1	100.0	23.0
1.0	0	99.6
1.0	1.0	96.2
1.0	10.0	83.3
1.0	100.0	11.1

[a] The virus present in the supernatant 6 days after infection was determined by an RT assay. The blank and uninfected control had means ± standard deviations of 842 ± 94 and 945 ± 94 dpm, respectively. Untreated infected controls (five replicates) had a mean ± standard deviation of 89.3 ± 9.8 kdpm per ml.

by using a chiral column, and the resolved (−)- and (+)-enantiomers were evaluated against HIV-1 in PBM cells. These compounds had retention times identical to that of the synthetic material obtained by using a stereoselective synthesis. The EC_{50}s of (−)-FTC and racemic FTC against HIV-1 in PBM cells were about 0.02 μM (slopes, 1.26 and 1.14, respectively), whereas the (+)-enantiomer had an EC_{50} of 0.28 μM (slope, 1.37). The order of magnitude for the HPLC-resolved enantiomers against HIV-1 was the same as that obtained with the synthetic enantiomers (Table 1), providing additional confirmation that the characterization of the different enantiomers was correct.

Racemic FTC was also effective against HIV-2 (Table 1). The resolved enantiomers of FTC were about 10-fold more potent against HIV-2 (strain ROD2) than against HIV-1 in PBM cells; the potency of the (−)-enantiomer was similar to that of AZT in these cells. The compounds were also effective in CEM and MT-4 cells infected with HIV-2 (strain Zy). These compounds appear to be selective for human retroviruses and were not active against herpes simplex virus type 1 or type 2 by a plaque assay in Vero cells, Friend murine retrovirus by a UV-XC assay in SC-1 cells, or feline leukemia virus-FAIDS in culture (data not shown). Moreover, FTC was found to be effective in culture against feline and simian immunodeficiency viruses, viruses with properties similar to those of HIV-1 and HIV-2 (24, 25, 47).

FTC and its enantiomers were also evaluated against AZT-resistant and -susceptible HIV-1 (Table 1). At the same multiplicity of infection, a two- to eightfold increased resistance was noted at the EC_{50} when the susceptibility of the pretherapy isolate (H112-2) was compared with that of the posttherapy AZT-resistant virus (G910-6) in PBM cells. This increase was not as great as that noted for AZT when the assay was performed with the same donor cells (Table 1). Similar results were obtained with BCH-189. The results indicated that all of the drugs tested with this pair of AZT-susceptible and -resistant viruses reached a statistically significant difference between the viral strains ($P < 0.05$). (−)-FTC and (−)-BCH-189 were also effective against a DDI-resistant isolate (strain M48). The nevirapine-resistant virus had a 150-fold increased resistance to nevirapine compared with that of the prototype LAV strain in HeLa $CD4^+$ cells. However, (−)-FTC was only 2.5-fold more resistant to the nevirapine-resistant virus (data not shown). Similar results were obtained when the assay was performed in acutely infected human PBM cells (Table 1).

The effects of increasing concentrations of 2'-deoxycytidine on the antiviral activity of (−)-FTC in human PBM cells infected with HIV-1 are presented in Table 2. 2'-Deoxycytidine prevented the anti-HIV-1 activity of (−)-FTC in a concentration-dependent manner. Other exogenous natural 2'-deoxy- and ribonucleosides, such as thymidine and cytidine, did not prevent the anti-HIV-1 activity of (−)-FTC when they were added up to concentrations of 100 μM. Similar results were obtained with the racemate (data not shown).

Previous work in our laboratory indicated that the toxicities of racemic BCH-189 and (+)-BCH-189 are more pronounced in CEM cells than they are in PBM or Vero cells (35). In contrast, FTC and its enantiomers did not inhibit cell proliferation in these cells when it was evaluated up to a concentration of 100 μM (Table 1).

Bone marrow toxicity has been demonstrated to be the limiting toxicity of certain nucleosides; therefore, the effects of FTC and its enantiomers on these cells were determined (Table 3). Racemic FTC and its (−)-enantiomer were significantly less toxic than AZT to human granulocyte macrophage precursor cells. Whereas AZT displayed a 50% suppression of colony formation at concentrations of 1.5 μM

TABLE 3. Relative effects of FTC and enantiomers on human myeloid (CFU-GM) and erythroid (BFU-E) progenitor cells by clonogenic assays

Compound	Cell	% of control (mean ± SD)[a] at:				IC_{50} (μM [mean ± SD])
		0.1 μM	1.0 μM	10 μM	100 μM	
(±)-FTC	CFU-GM	91 ± 8	80 ± 4	69 ± 6	34 ± 7	34 ± 12
	BFU-E	100 ± 9	90 ± 7	77 ± 6	55 ± 4	167 ± 56
(−)-FTC	CFU-GM	80 ± 11	79 ± 9	77 ± 10	36 ± 11	50 ± 30
	BFU-E	93 ± 12	92 ± 19	68 ± 4	36 ± 12	38 ± 15
(+)-FTC	CFU-GM	85 ± 14	77 ± 11	47 ± 10	15 ± 4	7.5 ± 3.9
	BFU-E	99 ± 9	95 ± 11	88 ± 10	34 ± 14	53 ± 31
AZT	CFU-GM					1.5 ± 1.2[b]
	BFU-E					0.6 ± 0.5[b]

[a] Results represent mean values of three separate experiments using different donors performed in triplicate. The correlation coefficient for the IC_{50}s was at least 0.93.

[b] Data were obtained from reference 43.

(Table 3), racemic FTC and (−)-FTC required a 22-fold or greater concentration to produce the same effect. Similar results were obtained in erythroid precursor cells (Table 3). It appears that racemic FTC and (+)-FTC were more toxic to granulocyte macrophage precursor cells than to erythroid precursor cells.

Biochemical studies. The interaction of the 5′-triphosphates of (±)-FTC and (−)-FTC and the HIV-1 RT in synthesis directed by $(rI)_n \cdot (dC)_{19-24}$ as a template indicated a competitive inhibition pattern with respect to dCTP, and an affinity which was similar to that for ddCTP was observed when a p66/51 heterodimer derived from the HXB2 clone was used. The values of K_i as determined by Dixon (8) plots were 3.9, 2.9, 9.0, and 5.1 μM for (±)-FTC-TP, (−)-FTC-TP, (+)-FTC-TP, and ddCTP, respectively. There was no significant difference between the K_is for ddCTP and (±)-FTC-TP. It appears that (−)-FTC-TP has a K_i two- to threefold lower than that of the (+)-enantiomer. Similar studies performed in a different laboratory, using a p66/51 HIV-1 RT derived from the HB10 clone and a $(rI)_n \cdot (dC)_{12-18}$ template primer, resulted in apparent K_is of 0.20 and 0.29 μM for (±)-FTC-TP and ddCTP, respectively. Under these conditions, the calculated mean K_m for dCTP was about 7.2 μM (range, 5.3 to 9.1 μM).

Additional studies were performed with the L74V/T215Y RT obtained by site-directed mutagenesis. The mutation at position 215 is associated with AZT-resistant virus, and that at position 74 is associated with DDI-resistant virus. The K_m for dCTP was increased from 21.0 ± 2.9 to 53.0 ± 72 μM. Similarly, the K_i for racemic FTC-TP was increased from 3.9 ± 0.99 to 28 ± 1.8 μM, and that for ddCTP was increased from 5.1 ± 0.7 to 75 ± 12 μM. ddCTP had an elevated K_i compared with that of racemic FTC with respect to the resistant enzyme. These data are consistent with the finding of a moderate decrease in the susceptibility of AZT-resistant virus to FTC (Table 1).

The incorporation of racemic FTC-TP into DNA was shown to be dose related when a sequencing method modified from that of Sanger et al. (28) over the range of 0.1 to 1 μM was used (Fig. 2). All the drug-related stops were independent of the natural stops, as demonstrated by the control lane without nucleotide analog. FTC-TP-induced stops on the DNA were limited to 2′-deoxycytidine positions, and no misincorporations of drug by HIV-1 RT p66/51 were noted. The assay spanned a 102-base region, and FTC-TP-related stops were evenly distributed throughout the template. The incorporation of FTC-TP mirrored that of ddCTP except at concentrations of 0.1 and 0.01 μM, where FTC demonstrated three more stops than ddCTP. The incorporation of 1 μM AZT-TP, which was used as a positive control, into the DNA template produced the anticipated T stops. It appears that incorporation of the cytidine analog instead of the natural nucleotide was about 49.2% ± 1.4% (mean ± standard deviation) successful, whereas for AZT-TP, a thymidine analog, the rate was about 35.5% ± 4.1% (Fig. 2). It should be noted that the intensity of the stops for FTC-TP at equimolar concentrations was greater than that for ddCTP.

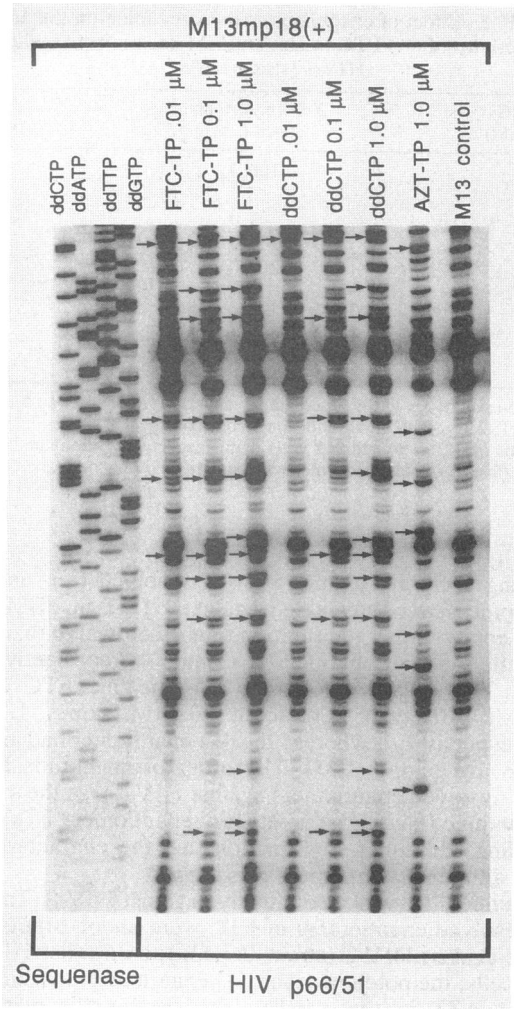

FIG. 2. Autoradiograph of the acrylamide gel from an experiment with the M13 forward primer on the complementary strand of M13mp18(+) at position −40. From the far left to right, the DNA sequence of M13mp18(+) is demonstrated with the Sequenase enzyme by using the inhibitors ddCTP, ddATP, ddTTP and ddGTP. Using the same primer-DNA complex and substituting with p66/51 RT (BioTechnology General, Inc.), the inhibitors FTC-TP, ddCTP, and AZT-TP were used at increasing concentrations. The M13mp18(+) control with no inhibitory compounds demonstrating natural stops of the enzyme is indicated in the rightmost lane. The arrows indicate strong stops which are not natural ones for the antiviral nucleotide analogs.

DISCUSSION

Enantiomeric natural nucleoside analogs with the unusual L-configuration have so far not been found in nature. Whereas enantiomers are always indistinguishable in achiral environments, their pharmacological and microbiological properties in chiral environments can be quite different. To date, with the exception of (−)-BCH-189 (6, 37, 38), no L-nucleoside has been found to be markedly more potent than its D-counterpart against HIV-1. Previous work on the metabolism of pyrimidine L-nucleosides has demonstrated that L-cytidine derivatives are deaminated in vivo to L-uridine derivatives (18). Concern that BCH-189 can be deaminated intracellularly by mammalian cytidine deaminase to the inactive uracil analog led us to synthesize a variety of nucleosides related to this lead compound. Racemic FTC was found to be the most potent anti-HIV-1 agent among the 5-substituted 3′-thiacytidine nucleosides synthesized (21, 37, 38). We hypothesized that the presence of fluorine may increase the lipophilicity of the drug, which may also increase its penetration into the central nervous system. In

addition, it was anticipated that replacement of the 5-position hydrogen by fluorine should accentuate the differences between these compounds and natural nucleosides and thereby enhance their selectivities toward HIV-1 RT. The finding that racemic FTC was active in cultures infected with HIV-1 provided the impetus to separate the (−)- and (+)-enantiomers by HPLC, to devise novel synthetic and enzymatic approaches to the synthesis of the two enantiomers, and then to determine the stereospecificities of the antiviral activities for these remarkable nucleosides.

The most potent compound, (−)-FTC, had activity comparable to that of AZT in HIV-1 (strain LAV)-infected PBM cells. No toxicity was apparent in the host PBM, CEM, or Vero cells when the compound was tested up to a concentration of 100 μM. The antiviral spectrum of this compound appeared to be limited to the human retroviruses HIV-1 and HIV-2 and the animal retroviruses simian and feline immunodeficiency viruses. Results of studies in TK^- CEM cells and metabolic studies with 2′-deoxycytidine kinase-deficient cells (38) suggest that the FTC isomers are probably phosphorylated by 2′-deoxycytidine kinase rather than by TK. Further evidence that this enzyme is germane was provided by prevention studies which indicated that the antiviral activity of FTC is reduced or abolished in the presence of high concentrations of 2′-deoxycytidine, but not with other natural 2′-deoxy- or ribonucleosides (Table 2). (−)-FTC is a selective anti-HIV-1 agent with a therapeutic index greater than 12,500 in PBM cells. This compound was at least 1 order of magnitude more potent than the (+)-enantiomer in primary human lymphocytes. The potent activity of the (−)-enantiomer in these cells may be related to its resistance to cytidine deaminase, whereas the (+)-isomer is a substrate for the mammalian enzyme (15, 37). However, it appears that tetrahydrouridine or 2′-deoxytetrahydrouridine, which are potent inhibitors of cytidine deaminase and cytidylate deaminase, did not enhance the anti-HIV-1 activity of (+)-FTC in PBM cells (data not shown). A more likely explanation for this difference is the fourfold lower affinity of (+)-FTC for mammalian 2′-deoxycytidine kinase (36). Moreover, (−)-FTC is rapidly and efficiently phosphorylated to the triphosphate form in human PBM cells (38) to a greater extent than the (+)-enantiomer is, which may explain the greater potency of (−)-FTC in these cells (data not shown). Racemic FTC and its enantiomers were less toxic than racemic BCH-189 or the (+)-BCH-189 in CEM cells. Overall, the virological data suggest that the (−)-enantiomer may have greater potency than (−)-BCH-189 in certain cell culture systems such as MT-4 cells and human monocytes (Table 1).

Like many other nucleosides, racemic FTC and its enantiomers were shown to be markedly more potent in PBM cells than in MT-4 cells. For example, the EC_{50} of (−)-FTC was 63-fold lower in PBM cells infected with HIV-1. This difference may be related to the different phosphorylation pattern for FTC and its enantiomers in PBM and MT-4 cells. To date, no significant difference in toxicity in various cells has been noted between the (−)-enantiomers of FTC and BCH-189. Whereas (−)-FTC and (−)-BCH-189 are active against HIV-1 in acutely infected cells, the compounds demonstrated no activity in chronically infected H9 cells or in preventing the reactivation of HIV-1 in OM-10.1 cells when they were evaluated up to a concentration of 100 μM (6, 13, 36).

Studies in bone marrow cells have been useful in predicting whether anemia will occur in humans (44). Toxicity to human bone marrow cells, especially cells of the erythroid lineage, is a limitation associated with the chronic administration of AZT (26). The therapeutic index, measured in terms of the ratio of toxicity (IC_{50}) to granulocyte-macrophage precursor cells to the median effective anti-HIV-1 (LAV) concentration in human PBM cells for (−)-FTC, was almost 6,000, which is significantly greater than the therapeutic index of AZT, which is 475. For erythroid precursor cells, the therapeutic index was over 4,900 for (−)-FTC. It is interesting that the (+)-enantiomer of FTC was more toxic to human myeloid cells than it was to erythroid progenitor cells; usually, the reverse is true for nucleosides (44). On the basis of the results obtained in granulocyte-macrophage precursor cells, one would predict that (+)-FTC is less desirable than the (−)-enantiomer as a candidate for clinical studies. In general, compounds in our clonogenic assay with IC_{50}s of ≤10 μM can express bone marrow toxicity in humans who use the drug on a long-term basis. It should be stated that the IC_{50}s of AZT obtained by other workers cannot be used for comparison with the data presented here since the results are dependent on various factors, including the type of serum used, the purity and source of the growth factors, as well as the duration of treatment. However, in general these assays are useful for ranking the order of toxicities of various nucleoside analogs (15).

(−)-FTC-TP was a selective competitive inhibitor of a heterodimer HIV-1 RT, with a K_i of 2.9 μM. (−)-FTC-TP and racemic FTC-TP had similar K_i values, which were about two- to threefold lower than the K_i values for (+)-FTC-TP. Analysis performed with a mutant HIV-1 RT containing the previously described AZT and DDI resistance mutations indicated that this enzyme is markedly more resistant to ddCTP than to (±)-FTC-TP. These findings suggest that RT is probably the primary site of action for FTC.

The use of a modified sequencing method (28) was effective for evaluating nucleoside analogs as potential chain terminators by using recombinant HIV-1 p66/51 RT (11, 39). In contrast to the end-labeling method (23), the modified method of Sanger et al. (28) allowed the comparison of naturally occurring resting sites along the template with the actual incorporation of the nucleoside. A stretch of 102 bases on the M13mp18(+) strand was used for the analysis (Fig. 2), making it easy to compare the number of incorporations for each drug with those for the other drugs. Racemic FTC-TP demonstrated a high number of stops at each concentration compared with those demonstrated by ddCTP and AZT-TP, indicating premature chain termination of DNA fragments. It is not clear whether the (−)-enantiomer, the (+)-enantiomer, or both nucleotide enantiomers present in racemic FTC are primarily responsible for the chain termination or the degree of reversibility of the incorporation. Similar studies with the individual enantiomers of FTC-TP at equimolar concentrations are planned.

It was interesting to find that both enantiomers of FTC were active against HIV-1 and HIV-2 in cell culture. It was also surprising to find that the (−)-enantiomeric form was generally more potent than the (+)-enantiomeric form. In fact, these compounds are good substrates for mammalian cytidine kinase and are rapidly phosphorylated in cells that express this enzyme (15, 37). A review of the current status of L-enantiomers of antiviral nucleosides was recently provided by several groups (2, 5, 6, 35, 41). Prior to those reports, the activities of most nucleosides were associated only with the D-isomers. It is significant that both (−)- and (+)-FTC, which are mirror images of each other, have significant antiviral activities at submicromolar concentra-

tions and that one enantiomer is more toxic than the other only in myeloid progenitor cells (Table 3). FTC and BCH-189 demonstrated some cross-resistance to AZT-resistant virus. The clinical significance of this cross-resistance will become apparent as these compounds are further developed. However, the lack of marked cross-resistance between (−)-FTC and AZT, DDI, and nevirapine suggests that these compounds could be used in combination since overlapping toxicity is unlikely (33).

The unexpected finding that certain L-isomers of nucleoside analogs of FTC and BCH-189 are potent and selective antiviral agents opens new approaches for the treatment of viral infections with nucleosides with the unusual L-configuration. Results of the studies described here provide a strong rationale for pursuing (−)-FTC as a compound that could be used to prevent and treat HIV-1 infections in humans. The finding that this compound also has potent anti-hepatitis B virus activity (9, 15) makes it a leading candidate for the advanced preclinical studies necessary to determine its safety and efficacy in humans infected with HIV-1, hepatitis B virus, or both viruses.

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