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Anti-Human Immunodeficiency Virus Activities of the β-L Enantiomer of 2′,3′-Dideoxycytidine and Its 5-Fluoro Derivative In Vitro

GILLES GOSSELIN,[1]* RAYMOND F. SCHINAZI,[2,3] JEAN-PIERRE SOMMADOSSI,[4] CHRISTOPHE MATHÉ,[1] MARIE-CHRISTINE BERGOGNE,[1] ANNE-MARIE AUBERTIN,[5] ANDRÉ KIRN,[5] AND JEAN-LOUIS IMBACH[1]

Laboratoire de Chimie Bioorganique, URA Centre National de la Recherche Scientifique 488, Université de Montpellier II, Sciences et Techniques du Languedoc, 34095 Montpellier Cédex 5,[1] and Université Louis Pasteur, Institut de Virologie de la Faculté de Médecine, Institute National de la Santé et de la Recherche Medicale Unité 74, 67000 Strasbourg,[5] France; Veterans Affairs Medical Center, Medical Research 151, Decatur, Georgia 30033[2]; Laboratory of Biochemical Pharmacology, Department of Pediatrics, School of Medicine, Emory University, Atlanta, Georgia 30322[3]; and Department of Pharmacology, Center for AIDS Research, Division of Clinical Pharmacology, University of Alabama at Birmingham, Birmingham, Alabama 35294[4]

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The L enantiomer of 2′,3′-dideoxycytidine (DDC) was recently shown to inhibit selectively human immunodeficiency virus type 1 (HIV-1) in vitro. In the current study, the potent anti-HIV activity of L-DDC was confirmed and extended to several HIV-1 and HIV-2 strains in various cell culture systems, including primary human lymphocytes and macrophages. Furthermore, its 5-fluoro congener, β-L-2′,3′-dideoxy-5-fluorocytidine (L-FDDC), was found to have more potent anti-HIV activity and a higher therapeutic index in acutely infected human peripheral blood mononuclear cells. These compounds had no marked activity against HIV-1 isolates resistant to the oxathiolane pyrimidine nucleosides (−)-β-L-2′,3′-dideoxy-5-fluoro-3′-thiacytidine [(−)-FTC] and (−)-β-L-2′,3′-dideoxy-3′-thiacytidine, but 3′-azido-3′-deoxythymidine (AZT)-resistant viruses were susceptible to L-DDC and L-FDDC. Cytotoxicity studies with human myeloid progenitor cells indicated that L-DDC and L-FDDC had median inhibitory concentrations comparable to those of AZT, DDC, and FDDC, but L-DDC and L-FDDC were significantly less toxic than AZT, DDC, and FDDC when erythroid progenitor cells were used. L-FDDC had the highest selectivity indices (6,000 and 9,000 for erythroid and myeloid progenitor cells, respectively) of all the compounds evaluated. Further preclinical development of L-FDDC is warranted in order to determine its potential usefulness in the treatment of HIV infections.

The synthesis and the biological evaluation of nucleoside analogs with the unnatural L configuration have been subjects of some interest, but until recently, the activities of most nucleosides were associated only with the D isomers (14). Newly published data indicate that L nucleosides deserve to be reconsidered as potential antiviral agents. Thus, on the one hand, synthetic β-L-thymidine has been shown to be selectively phosphorylated by herpes simplex virus type 1 thymidine kinase and to markedly reduce herpes simplex type 1 replication in HeLa cells (41). Although initially reported as having no antiviral activity (24), β-L-2′,3′-dideoxycytidine, the mirror image of 2′,3′-dideoxycytidine (DDC; or zalcitabine or Hivid), was shown by Mansuri et al. (22) to possess moderate activity against human immunodeficiency virus (HIV) in CEM cells. Moreover, the unexpected finding that the (−)-β-L-(2*S*,4*S*)-dioxolanylcytosine [(−)-BCH-204 (3); Fig. 1; X = H] (18, 19) and the (−)-β-L-(2*R*,5*S*)-1,3-oxathiolanylcytosine [(−)-BCH-189 or (−)-β-L-2′,3′-dideoxy-3′-thiacytidine (3TC) (15, 42); Fig. 1; Y = H] (2, 5, 16, 34) enantiomers exhibited more potent anti-HIV activities than the corresponding racemates or D isomers provides a strong rationale for studying the mirror images of other previously described D enantiomers. Among them are L-DDC and β-L-2′,3′-dideoxy-5-fluorocytidine (L-FDDC), since studies of the structure-activity relationship of pyrimidine nucleosides modified at the 5 position indicated that their potencies can be preserved or increased by having this position modified with a halogen. For instance, when the 5 position of DDC was substituted with a fluorine atom, both anti-HIV activity and potency were retained (17, 38). On the other hand, in the 2-hydroxymethyl-1,3-dioxolanyl (18) and -oxathiolanyl (16, 36) series, the β-L-5-fluorocytosine derivatives (Fig. 1; X and Y = F) were found to be the most potent anti-HIV compounds among those tested, although the dioxolanyl analog also demonstrated some toxicity. It is noteworthy that the β-L-enantiomer of 5-fluoro-1-[2-(hydroxymethyl)-1,3-oxathiolan-5-yl]cytosine [(−)-FTC] (Fig. 1; Y = F) was also found to be a potent inhibitor of hepatitis B virus replication, while the β-D-isomer was found to be considerably less active against hepatitis B virus (7).

Here, we report the anti-HIV activities, coresistance profiles, and cytotoxicities of enantiomerically pure L-DDC and L-FDDC (Fig. 1).

MATERIALS AND METHODS

Compounds. The synthesis of L-DDC and L-FDDC, which were stereospecifically obtained from L-xylose by a multistep reaction sequence, has been reported elsewhere (12). These compounds were characterized on the basis of their physical properties (melting points, and optical rotations; for L-DDC, melting point, 220 to 222°C; $[\alpha]^{20}_D$, −103.6 [c 0.8, methanol]; for L-FDDC, melting point, 158 to 160°C; $[\alpha]^{20}_D$, −80.0 [c 1.0, dimethyl sulfoxide]) and spectroscopic properties (UV, ^{1}H nuclear magnetic resonance, fast atom bombardment mass).

* Corresponding author. Mailing address: Laboratoire de Chimie Bioorganique, Université de Montpellier II, Sciences et Techniques du Languedoc, Case Courrier 008, Place Eugène Bataillon, Montpellier 34095-Cedex 05, France. Phone: (33) 67.14.38.55. Fax: (33) 67.04.20 29.

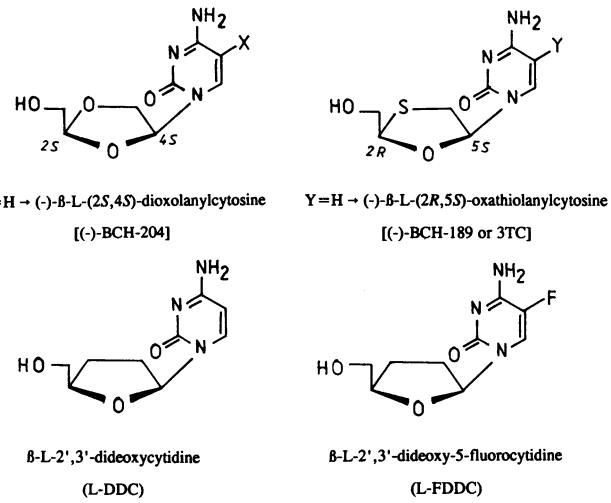

FIG. 1. Structures of (−)-BCH-204, (−)-BCH-189 (3TC), L-DDC, and L-FDDC.

Their purities were ascertained by combustion analyses and high-pressure liquid chromatography. DDC and 3′-azido-3′-deoxythymidine (AZT) were purchased from Intsel Marsing France (Paris). FDDC (17, 39) was kindly provided by V. Marquez (Laboratory of Medicinal Chemistry, National Institutes of Health, Bethesda, Md.).

Cell cultures and virus strains. The human T-cell lines CEM-SS (23), CEM-TK$^-$ (provided by D. Farquhar and W. Plunkett, University of Texas), MT-2 and MT-4 (13), the hybrid B- and T-cell line CEM X 174 (32), and the U-937 cell line of histiocytic origin (43) were propagated in RPMI 1640 medium supplemented with 2 mM glutamine and 10% fetal calf serum (FCS; heated for 30 min at 56°C) in an air–5% CO_2 incubator at 37°C. Human peripheral blood mononuclear (PBM) cells from healthy donors who were seronegative for HIV type 1 (HIV-1) were isolated by centrifugation and were propagated as described previously (36). Human macrophages were obtained by in vitro differentiation of monocytes which were isolated by centrifugal elutriation of PBM cells that were prepared as described above. An additional step of purification was obtained by plating the suspension of monocytes (in RPMI 1640 medium and 10% heat-inactivated FCS) on culture dishes and removing nonadherent cells after 2 h. The monolayers of monocytes were then cultured in AIM V (Gibco Laboratories, Eragny, France)–2 mM glutamine–100 U of granulocyte macrophage (GM) colony-stimulating factor (Genzyme; Dako S.A., Trappes, France) per ml, and the medium was changed after 4 days. After 1 week, the monocytes-macrophages were collected with a rubber policeman and were distributed in 48-well plates (Costar 3548) at a concentration of 3.5×10^5 cells per 250 μl of AIM V per well.

The virus isolates used were HIV-1 LAI, formerly HIV-1 BRU (1, 45); human T-cell lymphotrophic virus type IIIB (HTLV-IIIB) (26); HIV-1 Bal (9); HIV-2 D 194 (20); AZT-resistant HIV-1 (isolate G 910-6 obtained after drug treatment and isolate H 112-2 obtained before drug treatment) (21), and simian immunodeficiency virus (SIV) mac 251 (6), which was kindly provided by R. Desrosiers (New England Regional Primate Research Center, Harvard University Medical School, Southborough, Mass.).

Several cell lines and virus strains were obtained through the AIDS Research and Reference Program, National Institutes of Health: CEM-SS from P. L. Nara (National Cancer Institute, Frederick, Md.), CEM X 174 from P. Cresswell (Division of Immunology, Duke University Medical Center, Durham, N.C.), MT-2 cells and AZT-resistant HIV-1 from D. Richman (Veterans Affairs Medical Center, San Diego, Calif.), and HIV-1 Bal from S. Gartner, M. Popovic, and R. Gallo (Laboratory of Tumor Cell Biology, National Institutes of Health, Bethesda, Md.).

Assays in MT-4 cells. Replication of HIV-1 in MT-4 cells was determined by measuring the reduction in the viabilities of the cells resulting from the infection and was quantitated as described previously (10, 11, 25, 27) by a colorimetric reaction based on the capacity of the cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. The assays were performed in triplicate.

Assays on other cell cultures. Production of virus particles was evaluated by measuring virion-associated reverse transcriptase activity in the culture supernatant as described below. U-937, CEM-SS, CEM X 174, and CEM-TK$^-$ cells were infected with HIV-1 (20 50% tissue culture infective doses measured on CEM-SS cells), HIV-2, or SIV mac 251. After 0.5 h of adsorption, the residual free virus was removed by centrifugation, cells of the four in vitro systems cited above were resuspended, respectively, at concentrations of 2×10^4, 4×10^4, 4×10^4, and 8×10^4 cells per ml in the medium indicated above, and the suspensions were distributed into microtitration plates (100 μl per well) containing 0.1 ml of different dilutions of the antiviral drugs in each well. The assays were performed in duplicate. Reverse transcriptase activity was measured after 5 days for CEM-SS cultures, 6 days for CEM-TK$^-$ cultures, 7 days for U-937 cultures, and 10 days for CEM X 174 cultures. In the U-937 and CEM X 174 cultures, 100 μl of medium was removed after 5 days and was replaced with fresh medium containing the same concentration of drug. Seven-day-old monocyte-macrophage cultures were infected with HIV-1 Bal (0.1 ml per well; reverse transcriptase activity, 1.5×10^4 cpm). After 2 h at 37°C, the unadsorbed virus was removed. Cells were washed and incubated at 37°C in the presence of various concentrations of drugs. The medium was changed every 5 days with the same drug concentration. Reverse transcriptase activity was measured after 15 days. The 50% inhibitory concentration was determined by the MTT dye reduction assay as described above for MT-4 cells.

Cross-resistance assays. PBM cells from healthy donors seronegative for HIV-1 and hepatitis B virus were isolated by a single-step Ficoll-Hypaque Sigma, St. Louis, Mo. discontinuous gradient centrifugation and were propagated as described previously (36). The well-characterized (−)-FTC and 3TC-resistant viruses were obtained as described previously (35). Details on the infection of cells and assessment of antiviral effects have been reported previously (36). A multiplicity of infection of 0.1, as determined by a limiting dilution method in PBM cells, was selected for the assays. The 50% effective concentrations were derived from the computer-generated median effect plot of the dose-effect data as described previously (33).

Human CFU-GM or burst-forming units erythroid (BFU-E) clonogenic assays. Human bone marrow cells were collected by aspiration from the posterior iliac crest of healthy volunteers by a protocol approved by the Institutional Review Board Committee at the University of Alabama at Birmingham. Cells were treated with heparin, and the mononuclear population was separated by Ficoll-Hypaque gradient centrifugation as described previously (40). The culture assays were performed by using a bilayer of soft agar or the methylcellulose method (40). McCoy's 5A nutrient medium supplemented with 15%

dialyzed fetal bovine serum (heat inactivated at 56°C for 30 min; GIBCO Laboratories, Grand Island, N.Y.) was used in all experiments. This medium was devoid of thymidine and uridine.

Human recombinant GM colony-stimulating factor (50 U/ml; Genzyme, Boston, Mass.) or erythropoietin (1 U/ml; Connaught, Swifwater, Pa.) was used as a colony-stimulating factor. After 14 to 18 days of incubation at 37°C in a humidified atmosphere of 5% CO_2 in air, colonies (>50 cells) were counted by using an inverted microscope. The clonogenic efficiency was between 0.1 and 0.2% in all experiments.

RESULTS

Antiviral and cytotoxicity assays. The antiviral activities of L-DDC and its 5-fluoro derivative were first evaluated on CEM-SS cells infected with HIV-1 LAI. In this assay, both compounds were potent and selective inhibitors of HIV-1 at submicromolar concentrations (Table 1). L-FDDC was about 10-fold more potent than L-DDC and of the same order of potency as DDC.

The anti-HIV-1 activities and low toxicities of the β-L-enantiomers were also confirmed in several cell lines with different virus strains including an HIV-1 strain resistant to AZT (Table 1). In all cases, L-FDDC was consistently more effective than L-DDC in blocking HIV-1 replication, and the potency of the L-5-fluoro derivative was generally of the same order of magnitude as that of DDC. The difference was particularly marked in the monocytic cell line U-937. When compared with DDC, the fluoro derivative L-FDDC had a higher selectivity index. In monocyte-derived macrophages infected with a macrophage tropic isolate, HIV-1 Bal, DDC was more potent than L-FDDC, and L-FDDC was as potent as AZT (Table 1).

The β-L enantiomers were also effective against HIV-2 D 194 in PBM cells (data not shown). To inhibit SIV mac 251 replication in CEM X 174 cells, L-FDDC was used at a higher concentration than DDC, but the toxicity of L-FDDC was also lower (Table 1).

Cross-resistance studies. The decreasing order of potency of the nucleosides in acutely HIV-1 LAI-infected human PBM cells was L-FDDC > AZT ≃ (−)-FTC > L-DDC ≃ 3TC > FDDC (Table 2). L-FDDC was consistently about 1 order of magnitude more potent than either AZT or (−)-FTC in this cell culture system. None of the nucleosides was toxic to human PBM cells. L-DDC and L-FDDC were highly cross-resistant with 3TC and (−)-FTC (Table 2). At the same multiplicity of infection, a greater than 1,500-fold increased resistance was noted at the 50% effective concentration when the susceptibility of the laboratory strain HIV-1 LAI was compared with those of the 3TC- or (−)-FTC-resistant viruses.

As anticipated, a high level of cross-resistance with these isolates was evident with 3TC and (−)-FTC (Table 2) (35). On the other hand, L-DDC and L-FDDC were effective against a well-characterized AZT-resistant virus (Table 1).

Studies with human bone marrow cells. The effects of L-DDC and L-FDDC were evaluated and compared with those of the natural D-configuration derivatives DDC, FDDC, and AZT on human myeloid and erythroid colony-forming cells (Table 3).

The two L derivatives inhibited the myeloid CFU-GM cell growth to the same extent as FDDC and AZT, while DDC was two- to threefold more inhibitory. In BFU-E precursor cells, large differences in the inhibitory activities (between 20- and 40-fold) were displayed between the two L-derivatives and AZT, FDDC, and DDC. Of note, L-DDC and L-FDDC

TABLE 1. Antiviral activities and cytotoxicities of L-DDC and L-FDDC (and DDC and AZT as reference compounds) in various cell systems

Cell system (virus)	DDC		L-DDC		L-FDDC		AZT	
	EC_{50} (μM)[a,b]	IC_{50} (μM)[a,c]	EC_{50} (μM)[a]	IC_{50} (μM)[a]	EC_{50} (μM)[a]	IC_{50} (μM)[a]	EC_{50} (μM)[a]	IC_{50} (μM)[a]
CEM-SS (HIV-1 LAI)	0.0325 ± 0.0025	50 ± 10	0.42 ± 0.13	87.0 ± 1.8	0.044 ± 0.006	85 ± 5	0.0023 ± 0.0003	>100
MT-4 (HIV-1 IIIB)	2.6 ± 0.1	>100	14.5 ± 4.6	82.5 ± 7.5	2.35 ± 0.05	70 ± 10	0.0235 ± 0.0035	32.5 ± 22.5
CEM-TK⁻ (HIV-1 LAI)	0.0019 ± 0.0002	1.13 ± 0.67	0.09 ± 0.02	25.5 ± 2.4	0.07 ± 0.03	27 ± 2	>100	>100
MT-2 (HIV-1 H112-2[d])	0.32	>100 (22)[e]	2.5	>100 (18)	0.75	>100 (16)	0.075	>100 (22)
MT-2 (HIV-1 G 910-6[f])	0.46 ± 0.24	>100 (22)	0.58 ± 0.22	>100 (18)	0.95 ± 0.25	>100 (16)	>53	>100 (22)
U-937 (HIV-1 IIIB)	0.0015 ± 0.0008	<0.95	0.10 ± 0.03	42.8 ± 12.5	0.0010 ± 0.006	25 ± 3	0.005 ± 0.0002	65 ± 0
Macrophages (HIV-1 Bal)	0.00005	67.5 ± 2.5	0.003 ± 0.001	90 ± 10	0.00057 ± 0.00006	>100 (22)	0.00049 ± 0.00032	>95 ± 5
CEM X 174 (SIV mac 251)	0.0023 ± 0.0013	21 ± 4.9	0.13 ± 0.06	62.3 ± 5.4	0.035 ± 0.002	>93.5 ± 6.5	0.019 ± 0.013	>86 ± 14

[a] Values are means ± standard error of the mean of data obtained from duplicate or triplicate experiments.
[b] EC_{50}, 50% effective concentration.
[c] IC_{50}, 50% inhibitory concentration.
[d] Susceptible to AZT.
[e] Values in parentheses are the percent reduction of viable cells at the indicated highest concentration tested.
[f] Resistant to AZT.

TABLE 2. Susceptibilities of (−)-FTC- and (−)-BCH-189 (3TC)-resistant HIV-1 to L-DDC, L-FDDC, (−)-FTC, 3TC, and AZT in human PBM cells

Compound	EC_{50} (μM)[a]			Fold increase in EC_{50}	
	HIV-1 LAI	(−)-FTC-resistant HIV-1	3TC-resistant HIV-1	(−)-FTC	3TC
L-DDC	0.07 ± 0.02	>100	>100	>1,429	>1,429
L-FDDC	0.0002 ± 0.0006	82.9 ± 1.6	11.8 ± 5.2	3.7×10^5	5.4×10^4
FDDC	0.019 ± 0.002	ND[b]	ND	ND	ND
(−)-FTC	0.0014 ± 0.0006	>100	ND	$>7.1 \times 10^4$	
3TC	0.05 ± 0.04	ND	>100		>2,000
AZT	0.0010 ± 0.0003	0.005 ± 0.004	0.007	5	7

[a] Values are means ± standard errors of the means of data obtained from triplicate to sextuplicate determinations by using different donor cells. EC_{50}, 50% effective concentration.
[b] ND, not determined.

exhibited similar median inhibitory concentrations in both myeloid and erythroid cell lineages.

DISCUSSION

To date, only one enantiomeric nucleoside analog, namely, 9-β-L-(+)-adenosine, which has plant growth-stimulating properties (30), has been found in nature. However, the recent finding that L-thymidine (41) and some synthetic β-L-nucleoside analogs in the dioxolanyl and oxathiolanyl series (7, 37) are phosphorylated by viral or cellular kinases and exhibit potent antiviral activities provided the impetus to examine other L-enantiomer nucleosides. Conflicting data on the anti-HIV-1 activity of L-DDC in the literature (22, 24) prompted us to stereospecifically synthesize and study this compound and its 5-fluoro derivative (Fig. 1).

When evaluated for their in vitro inhibitory effects on the replication of human retroviruses HIV-1 and HIV-2 and the animal retrovirus SIV in several cell systems, the newly synthesized compounds L-DDC and L-FDDC demonstrated moderate and potent activities, respectively (Tables 1 and 2). The anti-HIV spectra of L-DDC and L-FDDC appeared to be similar to those of (−)-FTC and 3TC, but L-FDDC had the greatest potency when tested in primary human lymphocytes acutely infected with HIV-1 LAI (Table 2).

L-DDC and L-FDDC were effective against AZT-resistant viruses. On the other hand, there was a high degree of cross-resistance between these L enantiomers and the corresponding oxathiolane nucleosides when (−)-FTC- and 3TC-resistant viruses were used (Table 2). These viruses were found to contain a Met to Val or Ile mutation at codon 184 in the HIV-1 reverse transcriptase region of the genome (4, 8, 35, 44). The results described here indicate that L-DDC and L-FDDC, which are structurally related to the oxathiolane nucleosides, which contain an endocyclic 3′-thio group instead of the 3′-methylene group (Fig. 1), probably interact in the same way as (−)-FTC and 3TC with HIV-1 reverse transcriptase once they are phosphorylated. It appears that there is a stereoselectivity at the binding site in relation to the sugar ring and the cytosine moiety, but the 3′-thio group is probably not essential for binding.

TABLE 3. Cytotoxic effects of L-DDC and L-FDDC compared with those of DDC and AZT on human myeloid (CFU-GM) and erythroid (BFU-E) progenitor cells by clonogenic assays

Compound	CFU-GM		BFU-E	
	IC_{50} (μM)[a]	Si[b]	IC_{50} (μM)	SI
L-DDC	2.0 ± 0.8	28.6	2.4 ± 0.5	34.3
L-FDDC	1.2 ± 0.8	6,000	1.8 ± 0.5	9,000
DDC	0.7 ± 0.2	1,100	0.05 ± 0.04	78.6
FDDC	1.08 ± 0.26	56.8	0.30 ± 0.04	15.8
AZT	1.3 ± 0.5	1,300	0.10 ± 0.08	100

[a] Values are means of data obtained from four separate experiments performed in triplicate by using cells from different donors. The toxicities of the compounds were assessed simultaneously by using cells from the same donor. The 50% inhibitory concentration (IC_{50}) was derived from least-squares linear regression analysis of the logarithm of drug concentration versus CFU-GM or BFU-E survival fraction.
[b] SI, selectivity index. The selectivity index was calculated by determining the ratio of toxicity (50% inhibitory concentration) to bone marrow cells and the anti-HIV LAI activity measured in human PBM cells (50% effective concentrations from Table 2).

Clonogenic assays with human bone marrow have been useful in predicting whether the selected nucleoside analog will be associated with myelosuppression when given as a therapeutic agent to patients with AIDS (39). By these assays, all five tested compounds exhibited inhibitory effects against human CFU-GM within the same order of magnitude, with DDC being slightly more toxic (Table 3). Of note, while AZT has been associated with neutropenia (29), this side effect is not usually a limiting factor with DDC treatment (31). This is probably because administration of low DDC dosages should result in exposure to concentrations below those necessary to affect hematopoietic cell lineages. Therefore, in vivo administration of L-DDC or L-FDDC may also be selective for hematopoietic cell lineages when given within an adequate dosage range. When measured in terms of the ratio of toxicity (50% inhibitory concentration) to GM precursor cells (CFU GM) to the median effective anti-HIV-1 LAI concentration in PBM cells, the selectivity indices were 29 and 6,000 for L-DDC and L-FDDC, respectively, which is significantly greater than that for AZT, which has a selectivity index of 1,300. In addition, L-DDC and L-FDDC were highly selective compared with AZT, DDC, and FDDC when exposed to erythroid precursor cells (BFU-E); the selectivity index of L-FDDC was 90-fold greater than that of AZT.

In summary, L-FDDC is a potent and selective anti-HIV compound in cell cultures. A growing concern in the evaluation of this new antiretroviral agent is the possibility that drug-resistant HIV-1 variants could emerge in patients on therapy, which would limit its clinical efficacy (28). In vitro studies with L-DDC and L-FDDC are ongoing to develop viruses resistant to these compounds.

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