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Pure Nucleoside Enantiomers of β-2′,3′-Dideoxycytidine Analogs Are Selective Inhibitors of Hepatitis B Virus In Vitro

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(−)-β-L-2′,3′-Dideoxycytidine (β-L-DDC), (+)-β-D-2′,3′-dideoxycytidine (β-D-DDC), (−)-β-L-2′,3′-dideoxy-5-fluorocytidine (β-L-FDDC), (−)-β-L-2′,3′-dideoxy-5-fluoro-3′-thiacytidine (β-L-FTC), and (+)-β-D-1,3-dioxolane-5-fluorocytidine (β-D-FDOC) were evaluated for their anti-hepatitis B virus (anti-HBV) activities in HBV-transfected human liver cells (2.2.15). The order of decreasing potency for the compounds at the 90% effect level was β-D-FDOC > β-L-FTC > β-L-FDDC ≈ β-L-DDC >> β-D-DDC. Inhibition of HBV in transfected liver cells by the cytosine nucleosides was selective. The β-L-nucleoside-5′-triphosphates were consistently more potent inhibitors of woodchuck hepatitis virus DNA polymerase than the corresponding natural β-D-enantiomers.

Although widespread vaccination against hepatitis B virus (HBV) is a worthwhile goal, there are several million chronic carriers for whom therapy is the only possibility for delaying or preventing the progression of disease. In addition, since transplanted livers can be reinfected with HBV, the need to develop effective and nontoxic anti-HBV compounds for the prevention of the destruction of liver tissue after transplantation is essential (11). Several drug therapies have been explored for the treatment of HBV infection. These include adenine arabinoside, interferons, thymosin, acyclovir, phosphonoformate, zidovudine, (+)-cyanidanol, levamasole, quinacrine, and most recently, 2′-fluoroarabinosyl-5-iodouracil (4, 7, 11, 17). The last seven drugs have been shown to be either largely unsuccessful at treating HBV infection or too toxic. (−)-β-L-2′,3′-Dideoxy-3′-thiacytidine (3TC; Lavamudine) and (−)-β-L-2′,3′-dideoxy-5-fluoro-3′-thiacytidine (β-L-FTC) are leading anti-human immunodeficiency virus type 1 (anti-HIV-1) and anti-HBV oxathiolane nucleoside candidates that promise to have low toxicities to humans and potent activities against those viruses in humans (3, 13, 18).

On the basis of the findings of Schinazi and colleagues (13) with β-L-FTC, novel nucleosides with the unnatural L configuration were synthesized. These nucleosides are structurally related to β-L-FTC and were evaluated as potential antiviral agents. The finding that β-L-FTC and related cytidine derivatives of L nucleosides can be phosphorylated by 2′-deoxycytidine kinase (15) prompted us to synthesize (−)-β-L-2′,3′-dideoxycytidine (β-L-DDC) and (−)-β-L-2′,3′-dideoxy-5-fluorocytidine (β-L-FDDC). The antiviral spectra of these analogs appeared to be similar to those of β-L-FTC and 3TC, with activities against the human retroviruses HIV-1 and HIV-2 and the animal retrovirus simian immunodeficiency virus (5, 6). The report that β-L-FTC and 3TC are also selective inhibitors of HBV (3) prompted us to evaluate β-L-DDC and β-L-FDDC as potential inhibitors of HBV in transfected human hepatoblastoma-derived HepG2 (2.2.15) cells. For comparison, natural (+)-β-D-2′,3′-dideoxycytidine (β-D-DDC), β-L-FTC, and (+)-β-D-1,3-dioxolane-5-fluorocytidine (β-D-FDOC) were included in the study. The work described here explored the structure-activity relationship of L-cytidine analogs in which the 3′-thia group was modified to a 3′-methylene or a 3′-oxo group (Fig. 1). The synthesis and biological activities of some of the cytidine analogs described herein (10, 19) were recently reported. (This work was first presented by our group at the International Society for Antiviral Research, Charleston, S.C., 27 February to 4 March 1994 [14]).

β-L-DDC and β-L-FDDC were stereoselectively synthesized as described by Gosselin et al. (5). The optical rotations, $[\alpha]_D^{20}$, for β-L-DDC and β-L-FDDC were −103.6 (*c* 0.8, methanol) and −80.0 (*c* 1.0, dimethyl sulfoxide [DMSO]), respectively. β-L-FTC, β-D-FDOC, and β-L-FDOC and the nucleoside triphosphates were synthesized by previously published methods (8, 13, 20). (+)-β-D-2′,3′-Dideoxy-5-fluorocytidine (β-D-FDDC) was generously provided by Victor Marquez (National Institutes of Health, Bethesda, Md.). The purities of the enantiomers were confirmed by chiral high-pressure liquid chromatography as described previously (20). Stock solutions (40 mM) of the test compounds were prepared in DMSO. The nucleoside-5′-triphosphates either were purchased from Sigma Co. (St. Louis, Mo.) or were synthesized as

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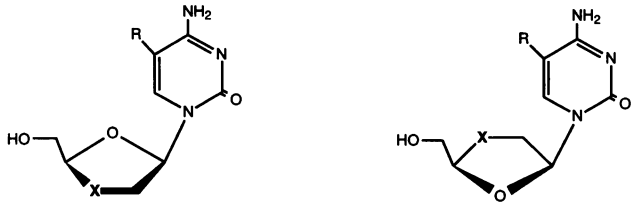

β-D-DDC: X = CH_2, R = H
β-D-FDDC: X = CH_2, R = F
β-D-FDOC: X = O, R = F

β-L-DDC: X = CH_2, R = H
β-L-FDDC: X = CH_2, R = F
β-L-FDOC: X = O, R = F
β-L-FTC: X = S, R = F

FIG. 1. Structures of β-L- and β-D-cytosine nucleoside enantiomers with antiviral activity in vitro.

described previously (13). The purities of these nucleotides, which exceeded 92%, were determined by anion-exchange high-pressure liquid chromatography.

The assays in HBV-transfected 2.2.15 cells were performed as described by Korba and Gerin (9), with minor modifications. The cells and the supernatant were harvested on day 9 after adding the compounds. Evaluation of the cytotoxicities of the compounds was conducted in 2.2.15 cells by measuring the uptake of neutral red dye, which was performed in 96-well flat-bottom cell culture plates. Cells were cultured and were treated with the test compounds on the same schedule used for the antiviral evaluations. Each compound was tested at four concentrations in triplicate cultures. The A_{510} of internalized dye was used for the quantitative analysis. 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 (13).

When the L-cytidine analogs and β-D-FDOC were evaluated in human liver HBV-transfected cells, the order of decreasing potency for the compounds at the 90% effect level was β-D-FDOC > β-L-FTC > β-L-FDDC ≈ β-L-DDC >> β-D-DDC (Table 1). Inhibition of HBV in 2.2.15 liver cells by all of the cytosine nucleosides appears to be selective, since none of the compounds was toxic when tested up to 200 μM. The order of decreasing selectivity index was β-D-FDOC > β-L-FTC > β-L-FDDC ≥ β-L-DDC >> β-D-DDC. β-L-FTC and β-D-FDOC had selectivity indices of greater than 600. Both β-L-FDDC and β-L-DDC were not only more potent but had higher selectivity indices than β-D-DDC (Table 1). The mechanisms of action of β-L-FDDC and β-L-DDC are probably due to inhibition of viral DNA polymerase and/or chain termination because of incorporation into an elongated DNA strand. L-DDC 5′-triphosphate and L-FDDC 5′-triphosphate, like (−)-L-FTC 5′-triphosphate, were shown to be potent DNA chain terminators by using HIV-1 reverse transcriptase (2, 12). Similar studies with HBV reverse transcriptase await the availability of the pure form of this enzyme.

A select number of β-L-cytosine nucleosides were evaluated against woodchuck hepatitis virus DNA polymerase. For this assay virus particles were concentrated from woodchuck hepatitis virus-positive serum, generously provided by B. Tennant (Cornell University, Ithaca, N.Y.), by using a 30% sucrose gradient centrifugation at 55,000 × *g* for 12 h. Pellets were resuspended in 400 μl of 50 mM Tris-HCl buffer (pH 7.6) containing 10% Nonidet P-40 and 100 mM 2-mercaptoethanol. Each reaction mixture (100 μl) contained 80 mM Tris-HCl (pH 7.6), 20 mM $MgCl_2$, 60 mM NH_4Cl, 250 μM dATP, 250 μM dGTP, 250 μM dTTP, and 1 μM [^{3}H]dCTP (60 Ci/mmol; New England Nuclear, Wilmington, Del.). The reaction was started by adding the disrupted virus particles. All of the assays were performed at 37°C for 3 h. Aliquots (50 μl) were spotted onto DE81 paper disks, washed in 125 mM Na_2HPO_4, dried, and counted. The IC_{50}s were determined from the dose-effect data as described above. The effects of the 5′-triphosphate derivatives of β-L-DDC and β-L-FDDC on woodchuck hepatitis virus DNA polymerase demonstrated that the β-L enantiomers were more potent inhibitors of this enzyme (fourfold or greater) than the corresponding β-D enantiomers. The IC_{50}s of the 5′-triphosphates of β-L-DDC, β-D-DDC, β-L-FDDC, β-D-FDDC, and (±)-DL-FTC (racemic compound) were 1.8, 7.5, 2.0, 10.0, and 1.3 μM, respectively (the variance for the data was less than 10%). These results are consistent with those in the recently reported work of Davis et al. (1) with β-L-FTC and 3TC.

Since a direct comparison of the pure β enantiomers of FDOC has never been reported, they were evaluated against HIV-1_{LAI} in acutely infected human peripheral blood mononuclear cells (13) and were found to be potent inhibitors, with EC_{50}s of 0.8 and 3 nM for the D and L enantiomers, respectively (data not shown). When tested against 3TC- and β-L-FTC-resistant viruses, the EC_{50}s of β-D-FDOC and β-L-FDOC increased by ≈ 10- and >100-fold, respectively. This suggests that the β-L enantiomer has a mechanism similar to those 3TC, β-L-FTC, β-L-DDC, and β-L-FDDC and that the β-L-nucleoside configuration, but not the substituent at the 3′ position (CH_2, O, or S; Fig. 1), is essential for conferring high-level resistance to oxathiolane nucleoside-resistant HIV-1 (12). The pure enantiomer of β-L-FDOC was markedly more toxic than β-D-FDOC in peripheral blood mononuclear, CEM, Vero, and human bone marrow cells (IC_{50}s, <2 μM; data not shown). Nevertheless, the data presented here provide the first example of highly potent enantiomers in which the β-D enantiomer is markedly less toxic than its β-L counterpart.

TABLE 1. Effect of enantiomers of cytosine nucleosides against HBV in transfected HepG2 (2.2.15) cells on day 9

Compound	HBV virion[a]		HBV RI[b]		Cytotoxicity (IC_{50} [μM])	Selectivity index ($IC_{50/EC_{90}}$)	
	EC_{50} (μM)	EC_{90} (μM)	EC_{50} (μM)	EC_{90} (μM)		Virion	RI
β-D-DDC	1.4 ± 0.5[c]	8.8 ± 2.1	2.7 ± 0.5	11.5 ± 2.2	218 ± 25	27	19
β-L-DDC	0.10 ± 0.01	3.6 ± 0.7	0.33 ± 0.03	5.9 ± 0.7	493 ± 64	136	84
β-L-FDDC	0.12 ± 0.01	2.8 ± 0.4	0.3 ± 0.03	4.8 ± 0.6	438 ± 57	156	91
β-D-FDOC	0.02 ± 0.003	0.2 ± 0.03	0.06 ± 0.01	0.23 ± 0.02	251 ± 23	1,255	1,091
β-L-FTC	0.04 ± 0.006	1.1 ± 0.1	0.16 ± 0.01	0.39 ± 0.22	746 ± 33	678	1,913

[a] The mean extracellular HBV DNA concentration on day 9 was 82 pg/ml.
[b] The mean replicative intermediates (RI) (intracellular HBV DNA) for untreated controls on day 9 was 85 pg/ml.
[c] Values are means ± standard deviations.

Of significance was the finding that β-L-DDC and β-L-FDDC had no effect on mitochondrial DNA synthesis when used at concentrations up to 100 μM (10, 16). On the basis of these data, further preclinical development of β-L-DDC, β-L-FDDC, and β-D-FDOC should be considered in order to determine their merits as potential antiviral agents for infections caused by HBV and HIV-1.

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