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RAPID COMMUNICATIONS

ANTIVIRAL ACTIVITY OF 2',3'-DIDEOXY-β-L-5-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*

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Abstract- 2',3'-Dideoxy-β-L-5-fluorocytidine (β-L-FddC) and 2',3'-dideoxy-β-L-cytidine (β-L-ddC), two nucleosides with "unnatural L-configuration," have been synthesized and found to have potent antiviral activity against hepatitis B virus (HBV) and human immunodeficiency virus type 1 (HIV-1) *in vitro* with very little toxicity. At 1 μM, both β-L-ddC and β-L-FddC inhibited the growth of HBV by more than 90%, while at the same concentration the D-configuration counterparts, 2',3'-dideoxy-β-D-cytidine (ddC) and 2',3'-dideoxy-β-D-5-fluorocytidine (β-D-FddC), did not show antiviral activity against HBV. The order of anti-HIV-1 activity was β-L-FddC > ddC; β-D-FddC > β-L-ddC. The dose-limiting toxicity of ddC is neuropathy which is believed to be caused by the inhibition of the synthesis of mitochondrial DNA. ddC severely inhibited the mitochondrial DNA synthesis of CEM cells yielding an IC_{50} value of 0.022 μM. Conversely, both β-L-FddC and β-L-ddC did not demonstrate any inhibition against mitochondrial DNA synthesis up to 100 μM concentration.
(Key words: HBV; HIV-1; ddC; 2',3'-dideoxy-β-L-5-fluorocytidine; 2',3'-dideoxy-β-L-cytidine; L-configuration; neuropathy)

Hepatitis B virus (HBV) is the causative agent of acute and chronic hepatitis, which affects nearly 300 million people worldwide [1]. Chronic infection has been shown to be closely related to the development of primary hepatocellular carcinoma [2,3]. Vaccination has been used for the prevention of HBV infection; however, at the present time, there are no effective drugs available for the treatment of HBV infection. Ueda *et al.* [4] and Yokota *et al.* [5] reported that 2',3'-dideoxycytidine (ddC) showed potent inhibition against hepatitis B virus replication. However, long-term usage of ddC has been associated with severe peripheral neuropathy, which was suggested to be caused by the depletion of mitochondrial DNA in host cells treated with the drug [6]. Therefore, it is highly desirable to modify the chemical structure of ddC to find new derivatives that could not only retain or possibly increase the activity of the parent compound, but also significantly reduce its toxicity.

In comparison to the natural β-D-nucleoside analogues, only a few studies have reported on the biological activity of L-nucleosides. Simuth and Holy [7] reported the interaction of some β-L-nucleoside derivatives, such as L-adenosine diphosphate (L-ADP), with bacterial polynucleotide phosphorylase. Recently, Spadari *et al.* [8] reported that L-thymidine is not recognized by human thymidine kinase, but functions as a specific substrate for the herpes simplex virus (HSV-1) viral enzyme and demonstrates anti-HSV-1 activity in HeLa cells. Mansuri *et al.* [9] also reported the synthesis and anti-human immunodeficiency virus type 1 (anti-HIV-1) activity of 2',3'-dideoxy-β-L-cytidine in CEM cells. In addition, Belleau *et al.* [10] and Soudeyns *et al.* [11] reported the synthesis and anti-

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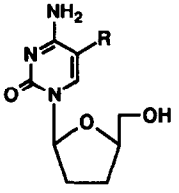
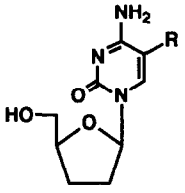
HIV-1 activity of 2',3'-dideoxy-3'-thiacytidine (BCH-189, (±)SddC). Doong *et al.* [12] first described that the racemic (±)SddC (BCH-189) demonstrated potent antiviral activity against HBV. Subsequently, Beach *et al.* [13] and Hoong *et al.* [14] reported the synthesis and anti-HIV-1 activity of (–)-2',3'-dideoxy-3'-thiacytidine [(–)SddC or (–)-3TC] and (–)-5-fluoro-2',3'-dideoxy-3'-thiacytidine [(–)FSddC or (–)-FTC] and their related enantiomers, respectively. Both (–)SddC [(–)-3TC] and (–)FSddC [(–)-FTC], which have the "L-configuration," showed potent antiviral activity against HIV and HBV in culture [15-18]. In this communication, we report the synthesis and potent antiviral activities of 2',3'-dideoxy-β-L-5-fluorocytidine (β-L-FddC) and 2',3'-dideoxy-β-L-cytidine (β-L-ddC) against HBV and HIV-1 along with very low toxicity. More detailed results will be published in a manuscript submitted elsewhere.

MATERIALS AND METHODS

β-L-FddC and β-L-ddC were synthesized by direct coupling of the respective silylated 5-fluorocytosine and cytosine with 1-*O*-acetyl-5-*O*-(*tert*-butyldimethylsilyl)-2,3-dideoxy-L-ribofuranose, followed by deblocking of the *tert*-butyldimethylsilyl protecting group.* The structures and physical and spectroscopic data of β-L-FddC and β-L-ddC are described as follows:



R = F (β-L-FddC)
R = H (β-L-ddC)



R = F (β-D-FddC)
R = H (β-D-ddC, ddC)

2',3'-Dideoxy-β-L-5-fluorocytidine (β-L-FddC) was isolated as white crystals: m.p. 147-149°; $[\alpha]_D$ -108° (c = 0.13, MeOH); UV (MeOH) λ_{max} 285 nm (ε 6481), λ_{min} 263 nm; UV (0.01 N HCl) λ_{max} 290 nm (ε 10990), λ_{min} 250 nm; UV (0.01 N NaOH) λ_{max} 283 nm (ε 7984), λ_{min} 260 nm; ^{1}H NMR (Me_2SO-d_6) δ 1.76-2.28 (m, 4 H, 2'-H and 3'-H), 3.49-3.57 (m, 1 H, 5'-H_A), 3.69-3.77 (m, 1 H, 5'-H_B), 3.98-4.04 (m, 1 H, 4'-H), 5.11-5.15 (t, 1 H, 5'-OH, D_2O exchangeable), 5.85 (m, 1 H, 1'-H), 7.40 and 7.62 (two s, 2 H, 4-NH_2, D_2O exchangeable), 8.26 (d, 1 H, 6-H, *J* = 7.4 Hz). Anal. Calcd. for $C_9H_{12}FN_3O_3 \cdot 0.3H_2O$: C, 46.07; H, 5.41; N, 17.91. Found: C, 45.72; H, 5.48; N, 17.95.

2',3'-Dideoxy-β-L-cytidine (β-L-ddC) was isolated as white solid: m.p. 194-196°; $[\alpha]_D$ -90.3° (c = 0.14, MeOH); UV (MeOH) λ_{max} 270 nm (ε 6979), λ_{min} 248 nm; UV (0.01 N HCl) λ_{max} 282 nm (ε 11965), λ_{min} 242 nm; UV (0.01 N NaOH) λ_{max} 273 nm (ε 8340), λ_{min} 250 nm; ^{1}H NMR (Me_2SO-d_6) δ 1.74-2.24 (m, 4 H, 2'-H and 3'-H), 3.49-3.65 (m, 2 H, 5'-H), 3.98-4.04 (m, 1 H, 4'-H), 4.96-5.00 (t, 1 H, 5'-OH, D_2O exchangeable), 5.67 (d, 1 H, 5-H, *J* = 7.4 Hz), 5.91 (m, 1 H, 1'-H), 7.01-7.06 (br s, 2 H, 4-NH_2, D_2O exchangeable), 7.87-7.90 (d, 1 H, 6-H, *J* = 7.4 Hz). Anal. Calcd. for $C_9H_{13}N_3O_3 \cdot 0.5CH_3OH$: C, 50.43; H, 6.67; N, 18.57. Found: C, 50.58, H, 6.32, N, 18.45.

The synthesis and anti-HIV-1 activity of β-L-ddC were reported previously by Mansuri *et al.* [9]; however, no physical properties, experimental details, spectroscopic data, or anti-HBV activity were reported in their paper.

Anti-HIV-1 assays. In a modification of a procedure by Mellors *et al.* [19], the compounds were tested in drug susceptibility assays for determining their effectiveness against HIV-1 in MT-2 cells. Drug-mediated inhibition of virus-induced cell toxicity was measured by the A_{595} of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma-M-2128). Triplicate wells of a 96-well tissue culture plate containing 1 x 10^4 MT-2 cells (AIDS-repository) per well were infected with HIV-1 (HTLV-IIIB Strain - R. C. Gallo) virus at a multiplicity of 0.1 $TCIC_{50}$/cell. MT-2 cells in RPMI 1640 medium supplemented with 10% dialyzed fetal bovine serum and 100 μg/mL Kanamycin were infected with virus and immediately added to serial dilution of the drug. After 5 days,

*The details of the syntheses of 2',3'-dideoxy-β-L-5-fluorocytidine (β-L-FddC), 2',3'-dideoxy-β-L-cytidine (β-L-ddC), and other related analogues have been submitted for publication elsewhere.

20 μL of MTT dye (2.5 mg/mL in PBS) was added per well. At the end of a 4-hr incubation period, 150 μL of acidified 2-propanol with 2% NP-40 nonionic detergent was added per well. After the crystals of dye dissolved (usually 1-2 days), the plates were read on a micro-plate reader. Using this MTT-dye reduction method [20], the percentage of protection can be calculated using the formula [(*a-b*/*c-b*) x 100] in which *a* = A_{595} of drug-treated virus-infected wells, *b* is the A_{595} of no-drug infected cells, and *c* is the A_{595} of the no-drug uninfected cells.

Anti-HBV assays. The biological activity of the compounds was assessed as described by Doong *et al.* [12]. The human hepatoma cell line carrying the HBV (designated 2.2.15), provided by Dr. G. Acs, was used in the study [21]. Six-day-old cultures were treated with various concentrations of the drug in the culture medium (Minimum Essential Medium with Earl's salts and 10% fetal bovine serum). The drug was left in the culture medium for a period of 3 days, after which period the medium was aspirated and fresh medium containing the same concentration(s) of the drug was added. At the end of the subsequent 3-day period, the culture medium was harvested and processed to obtain the virions by the polyethylene glycol precipitation method [12]. The viral DNA recovered from the secreted particles was subjected to Southern analysis. Inhibition of the viral DNA was determined from drug-treated versus control cultures not treated with the drug.

RESULTS AND DISCUSSION

β-L-FddC and β-L-ddC were tested *in vitro* against HIV-1 and HBV, and compared with 2',3'-dideoxy-β-D-cytidine (β-D-ddC, ddC), which is currently a clinically used drug for the treatment of AIDS, and 2',3'-dideoxy-β-D-5-fluorocytidine (β-D-FddC), which was synthesized and reported to have the same level of anti-HIV-1 activity as ddC by Kim *et al.* [22]. The order of anti-HIV-1 activity was: β-L-FddC > ddC; β-D-FddC > β-L-ddC. At 1 μM, both β-L-ddC and β-L-FddC inhibited the growth of HBV by more than 90%. Conversely, at the same concentration (1 μM), both ddC and β-D-FddC did not inhibit the growth of HBV at all. These findings are summarized in Table 1.

Table 1. Inhibitory effects of ddC, β-L-ddC, β-D-FddC, and β-L-FddC against the growth of HIV-1, HBV, and CEM cells *in vitro*

	% Inhibition			IC_{50}[f] (μM)
Compound	HIV-1[a] (0.4 μM)[c]	HIV-1[a] (1.2 μM)[d]	HVB[b] (1 μM)[e]	CEM
ddC	25	63	none	28
β-L-ddC	9	17	>90[g]	70
β-D-FddC	23	52	none	2
β-L-FddC	35	96	>90[g]	67

[a]HIV-1 assays were performed using a viral multiplicity of 0.1 $TCIC_{50}$/cell. [b]Percent inhibition was determined based on the densitivity of autoradiographs following the procedure of Doong *et al.* [12]. [c]The concentration of drug used was 0.4 μM. [d]The concentration of drug used was 1.2 μM. [e]The concentration of drug used was 1 μM. [f]The concentration of drug used to inhibit replication of CEM cells by 50% after 72 hr of incubation. [g]Quantitative analysis of more than 90% inhibition was no longer accurate.

The cytotoxicity of ddC against CEM cells in culture was ~ 2.5 times more toxic than that of β-L-ddC with IC_{50} values of 28 and 70 μM, respectively. However, β-D-FddC was ~ 34 times more toxic than β-L-FddC against CEM cells with the corresponding IC_{50} values of 2 and 67 μM.

The dose-limiting toxicity of ddC is severe neuropathy which may be caused by the inhibition of the synthesis of mitochondrial DNA. ddC severely inhibited the mitochondrial DNA synthesis (IC_{50} 0.022 μM) of CEM cells. On the contrary, β-L-FddC and β-L-ddC showed no inhibition against mitochondrial DNA synthesis at a concentration

of 100 μM.*

Unlike ddC and β-D-FddC, β-L-ddC and β-L-FddC have an "unnatural L-configuration" and showed unexpected potent anti-HIV-1 and -HBV activities, meriting further development as potential anti-HIV and anti-HBV agents.

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*The methodology for the determination of the effects of these compounds against CEM cells and mitochondrial DNA synthesis will be reported in detail in a paper submitted for publication elsewhere.