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RAPID COMMUNICATIONS

POTENT AND SELECTIVE IN VITRO ACTIVITY OF 3'-DEOXYTHYMIDIN-2'-ENE (3'-DEOXY-2',3'-DIDEHYDROTHYMIDINE) AGAINST HUMAN IMMUNODEFICIENCY VIRUS

Tai-Shun Lin*, Raymond F. Schinazi# and William H. Prusoff*

*Department of Pharmacology, Yale University School of Medicine, New Haven, CT 06510;
#Veterans Administration Medical Center (Atlanta) and Department of Pediatrics, Emory University School of Medicine, Atlanta, GA 30303, U.S.A.

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Various compounds have been reported to inhibit in cell culture the replication of the retroviruses including human immunodeficiency virus (HIV-1, HTLV-III/LAV) [1-21], and of these 3'-azido-3'-deoxythymidine (AZT) [16-20] and several 2',3'-dideoxynucleosides (ddNs) [21] are very potent. Among the nucleosides, 2',3'-dideoxycytidine (ddCyd; d2C) is the most potent inhibitor with complete inhibition of the cytopathic effect in ATH8 cells at a concentration of 0.5 μM [21]; in our laboratory it has a potency similar to AZT but appears to be less toxic to uninfected peripheral blood mononuclear (PBM) cells [22]. The mechanism(s) by which 2',3'-dideoxynucleoside-5'-triphosphate (ddNTP) inhibits DNA polymerase is not clear. Incorporation of ddNTP onto the 3'-end of a growing DNA chain would prevent further elongation since ddNTPs do not have a hydroxyl function in the 3'-position. That such chain termination may be involved in inhibition of retroviral DNA polymerases is suggested by the short size of the DNA chains synthesized in the presence of ddNTP [23]. Under conditions of primer excess, the mammalian cellular DNA polymerases α, β, and γ are inhibited competitively by ddNTP which could be due either to simple competition at the dNTP binding site of the polymerase or to incorporation into DNA with subsequent termination of chain growth. Specificity of inhibition may be achieved based on the finding of Waqar et al. [23] that ddNTP is more inhibitory to retroviral reverse transcriptase than cellular DNA polymerase α, and the report by Kutateladze et al. [24] that the 3'-O-methyl derivative of 2'-dNTPs is a specific inhibitor of reverse transcriptase. The metabolism of d2C in uninfected and HIV infected human and non-human cells was investigated by Cooney et al. [25] and with the exception of a deoxycytidine kinase deficient cell line (P388/AAC), all are capable of converting d2C to the mono-, di- and triphosphates. In addition the 5'-diphosphocholine complex of d2C is also formed in several of the cell lines [25]. 3'-Azido-3'-deoxythymidine as the triphosphate derivative was found by Furman et al. [26] to be more inhibitory to reverse transcriptase than to cellular DNA polymerase α. Because 2',3'-dideoxycytidine is a potent inhibitor of HIV, both Balzarini et al. [27] and Lin et al. [22] investigated independently the antiviral activity of the 2',3'-unsaturated analog of 2',3'-dideoxycytidine. Both groups found 2',3'-dideoxy-2',3'-didehydrocytidine (2',3'-dideoxycytidin-2'-ene; d4C) to be a potent inhibitor of HIV-1. Lin et al. [28] previously reported the antiviral activities of these two analogs against the Moloney - murine leukemia virus and found the EC_{50} using the XC-assay to be 4.0 μM for d2C and 3.7 μM for d4C. Lin et al. [22] reported an EC_{50} of about 0.005 μM for the inhibition of HIV-1 in human peripheral blood mononuclear cells by d4C, whereas Balzarini et al. [27] found that this compound at 0.1 μM exerts about 40% inhibition of the cytopathogenicity of HIV-1 in ATH8 cells and complete protection at 0.5 μM. The differences in potency may be related to the strain of virus, the multiplicity of infection (MOI) and the host cell.

Mitsuya et al. [29] recently reported that 2',3'-dideoxy-2',3'-didehydroadenosine (d4A) is moderately active in preventing an HIV-1 induced cytopathic effect in ATH8 cells, but is also toxic at slightly higher concentrations to uninfected cells. Balzarini et al. [27] reported that the corresponding thymidine analog has activity similar to d4A, and supporting data for the activity of d4T in MT4 cells were presented by Baba et al. [30] which is in agreement with the present report.

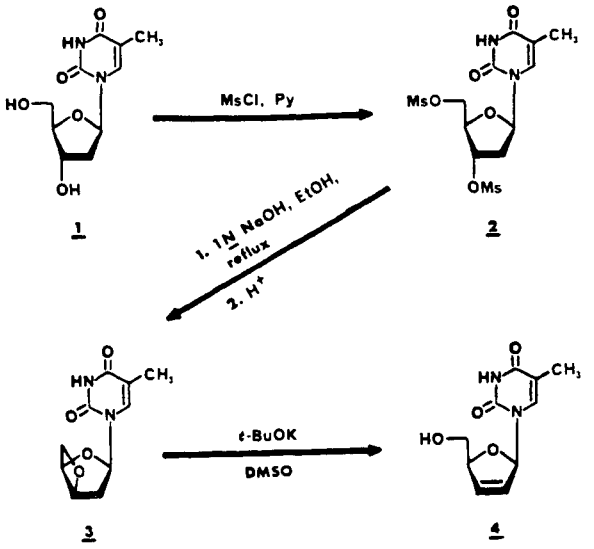
The present report describes and compares the antiviral activities of 3'-deoxythymidine (d2T) and its unsaturated analog 3'-deoxy-2',3'-didehydrothymidine (d4T), against HIV, as evaluated by an assay of reverse transcriptase present in solubilized virus obtained from supernatant of virus infected PBM cells. The effects of these compounds on uninfected cells are also reported.

SYNTHETIC METHODOLOGY

3'-Deoxythymidin-2'-ene (3'-deoxy-2',3'-didehydrothymidine; d4T) (4) was synthesized by the methodology of Horwitz et al. [31] with minor modifications. Treatment of thymidine (1) with excess methanesulfonyl chloride in pyridine at 0°C gave the corresponding disulfonate 2. Refluxing compound 2 with 1 N NaOH solution in ethanol produced the 3',5'-cyclic ether 3. Treatment of compound 3 with potassium t-butoxide in dry dimethyl sulfoxide (DMSO) yielded the desired 2',3'-unsaturated derivative 4. The synthesis is shown

in Scheme 1.

Scheme 1



3'-Deoxythymidin-2'-ene (3'-deoxy-2',3'-didehydrothymidine) 4. A solution of the cyclic ether (3, 8.64 g, 38.4 mmol) in 240 ml of dried DMSO containing 8.70 g (76.4 mmol) of potassium t-butoxide was stirred at room temperature for 2 hr. The reaction mixture was neutralized to ~ pH 7 with ethanolic acetic acid, and the solution was then evaporated to dryness at ~ 50° under reduced pressure. The residue was triturated with several portions of hot acetone. The insoluble materials were removed by filtration, and the filtrate was evaporated to dryness. The residue was eluted through a silica gel column ($CHCl_3$-EtOH, 2:1) to yield 6.5 g (76%) of product: m.p. 158-160°; NMR (Me_2SO-d_6) δ 1.82 (s,3H,5-CH_3), 3.53 (m, 2H, 5'-H), 4.80 (m, 1H, 4'-H), 4.96 (t, 1H, 5'-OH, D_2O exchangeable), 5.90 (m, 1H, 3'-H, vinyl), 6.40 (m, 1H, 2'-H, vinyl), 6.82 (m, 1H, 1'-H), 7.67 (s, 1H, 6-H).

ANTIVIRAL ASSAY IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

Three-day-old mitogen-stimulated human peripheral blood mononuclear cells (10^6 cells/ml) were infected with HIV-1 (strain LAV) at a concentration of about 100 IC_{50} per ml [32] and cultured in the presence and absence of various concentrations of d2T and d4T. Five days after infection, the supernatant was clarified and the virus pelleted at 40,000 rpm. The reverse transcriptase activity in the disrupted virus was determined. The methods used for culturing the PBM cells, harvesting the virus, and determining the reverse transcriptase activity were those described by McDougal et al. [32] and Spira et al. [33]. The data shown in Fig. 1 clearly indicate that the two compounds were both active with approximate EC_{50} values of 0.17 μM for d2T and 0.0088 μM for d4T, as determined by the Median Effect Method [34]. 2'-Deoxythymidine has been reported previously to inhibit ATH8 cell cytopathic effect at concentrations as high as 200 μM [21]. The difference between this report and our data in PBM cells may be related to differences in metabolism of the drug, or pool sizes of thymidine nucleotides, or both.

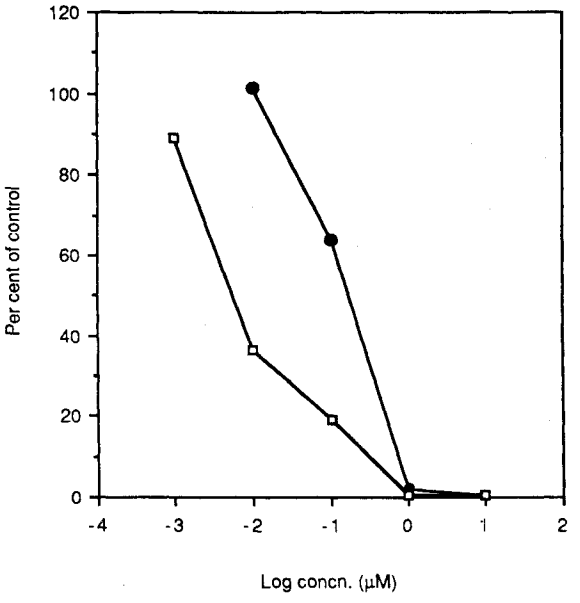


Fig. 1. Effects of 3'-deoxythymidine (d2T) (●) and 3'-deoxythymidin-2'-ene (3'-deoxy-2',3'-didehydrothymidine; d4T) (□) on the replication of HIV-1 in human PBM cells. The mean of triplicate counts (± S.D.) for the virus control was 176 x 10^3 ± 22 x 10^3 dpm/ml. The blank and negative control (no virus or drug) were 260 and 1046 dpm, respectively. A positive control for the reverse transcriptase assay was included (152 x 10^3 dpm).

Comparison of the EC_{50} values of d2T and d4T with those of the corresponding deoxycytidine analogs d2C (0.011 μM) and d4C (0.005 μM) shows that the unsaturated nucleosides (d4C and d4T) were more potent than the saturated analogs (d2C and d2T). Whereas the two unsaturated analogs had similar antiviral activities, d2C was about 15-fold more potent than d2T.

The effect of delayed treatment with the nucleoside analogs was examined by adding the drugs on day 0, 1, 2, 3 or 4 after virus infection (Table 1). The results suggest that d2C was the most effective drug at preventing virus replication; a 64% inhibition of virus replication was apparent when the drug was added on day 3 after virus infection. The relative order of prevention of virus replication was d2C > AZT > d4T > d4C > d2T.

Table 1. Effect of delayed treatment with nucleotide antivirals on the replication of HIV-1 in human PBM cells[a]

Day drug added[b]	Treatment - Percent Inhibition				
	d2T(1 μM)	d4T(0.1 μM)	d2C(0.1 μM)	d4C(0.1 μM)	AZT(0.1 μM)
0	70.9	71.8	91.6	73.3	94.0
1	49.7	62.8	83.6	34.8	89.0
2	0	50.0	78.7	30.4	79.0
3	0	27.0	64.3	15.5	38.0
4	0	0	ND[c]	ND	ND

(a) The mean of duplicate counts for the virus control was 340 x 10^3 dpm/ml. The blank and negative control (no virus or drug) were 306 and 1,080 dpm, respectively.
(b) Drug from a 100 μM stock solution was added on different days after virus infection.
(c) Not determined.

The effects of the drugs on the growth of uninfected human PBM cells were also established. Mitogen-stimulated PBM cells (3.8×10^5 cells/ml) were cultured in the presence and absence of drugs under the same conditions as those used for the antiviral assays described above. The cells were counted daily for 5 days using the trypan blue exclusion method. The data in Fig. 2 indicate that d4T at 10 μM did not inhibit the replication of human PBM cells, but at 100 μM a 70% inhibition was observed. In contrast, d2T at 100 μM was not inhibitory to the replication of PBM cells.

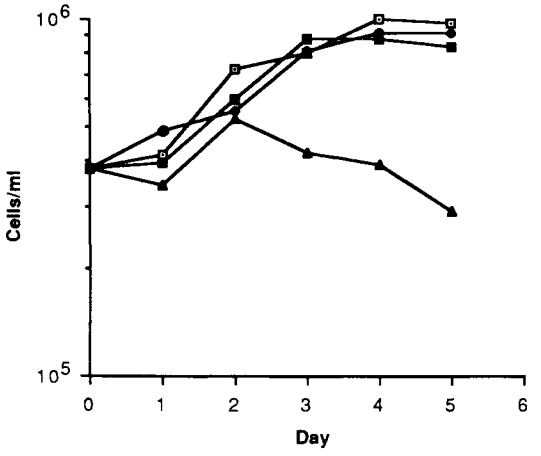


Fig. 2. Effects of 3′-deoxythymidine (d2T) and 3′-deoxythymidin-2′-ene (3′-deoxy-2′,3′-didehydrothymidine, d4T) on the growth of human PBM cells relative to control. Key: Control (□); d2T, 100 μM (●); d4T, 100 μM (▲); and d4T, 10 μM (■).

The results for the different compounds relative to AZT and related cytidine analogs [22] are described in Table 2. The variation of the data was less than 20 percent when cells from different donors were used.

Table 2. Summary of median effective concentration (EC_{50}) and toxicity of nucleoside analogs

Compound	EC_{50}, (nM) on day 5	Percent inhibition of uninfected cells on day 5 (100 μM)
d2C	11	0
d2T	170	0
d4C	5	46
d4T	9	70
AZT	2	68

CONCLUSION

Reviews of the various compounds evaluated for their activities against HIV, as well as discussion of the AIDS problem in general have been presented [35-38]. The data above clearly indicate that d4T is a more potent inhibitor of HIV than d2T in human peripheral blood mononuclear cells in vitro. Although d2T was at least 10-fold less toxic than d4T to uninfected cells, the unsaturated analog (d4T) was about 19 times more potent as an antiviral agent. d4T (0.1 μM) was markedly more effective in inhibiting viral replication than d2T (1.0 μM) when treatment was delayed by 1 or 2 days after infection.

The therapeutic index of the unsaturated analog of thymidine (d4T) appears to be close to 5000. Thus, the potent activity of d4T against HIV-1 and its very high therapeutic index merit additional studies in experimental animals and, hopefully, in the therapy of patients with AIDS and related disorders.

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