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ANTIVIRAL ACTIVITY OF 2',3'-DIDEOXYCYTIDIN-2'-ENE (2',3'-DIDEOXY-2',3'-DIDEHYDROCYTIDINE) AGAINST HUMAN IMMUNODEFICIENCY VIRUS *IN VITRO*

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Acquired immunodeficiency syndrome (AIDS) is generally accepted to be a consequence of infection with the retrovirus variously termed human T-lymphotropic virus type III (HTLV-III), lymphadenopathy-associated virus (LAV), AIDS associated retrovirus (ARV), or human immunodeficiency virus (HIV). A number of compounds have demonstrated antiviral activity against this virus which include HPA-23 (1,2), interferons (3), ribavirin (4), phosphonoformate (5,6), ansamycin (7), suramin (8-10), imuthiol (11), penicillamine (12), rifabutin (13), AL-721 (14), 3'-azido-3'-deoxythymidine (15-19), and more recently various 2',3'-dideoxynucleosides (20) of which 2',3'-dideoxycytidine (ddCyd) is the most potent. A review of these and other compounds evaluated for their activities against HIV, as well as a discussion of the AIDS problem in general, has been presented (21).

This report describes the antiviral activity against HIV of 2',3'-dideoxycytidin-2'-ene (2',3'-dideoxy-2',3'-didehydrocytidine; D4C), and compares the antiviral activity with that of 2',3'-dideoxycytidine and 3'-azido-3'-deoxythymidine (AZT, BW A509U). The 2',3'-dideoxycytidin-2'-ene, 2',3'-dideoxycytidine, and 3'-azido-3'-deoxythymidine were first synthesized by Horwitz *et al.* (22,23); however, a different route was adopted for the preparation of the two cytidine compounds using, as starting material, 2',3'-dideoxyuridin-2'-ene (2',3'-dideoxy-2',3'-didehydrouridine) and 2',3'-dideoxyuridine.

SYNTHETIC METHODOLOGY

Compound 1 (22) acetylated with acetic anhydride in pyridine gave the corresponding acetate 2 which was then treated with 4-chlorophenyl phosphorodichloridate and 1,2,4-triazole in pyridine at room temperature to yield the 4-triazolylpyrimidinone derivative 3. Subsequent treatment of the 4-triazolylpyrimidinone derivative with aqueous ammonia in dioxane (1:3) for several hours and then methanolic ammonia overnight at room temperature yielded compound 4. The synthesis is shown in Scheme I.

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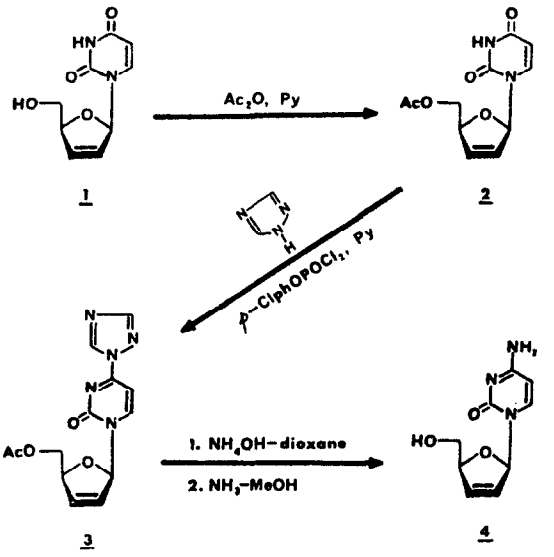
Scheme 1



Acetic anhydride (1.00 g, 10.0 mmol) was added slowly to a stirred solution of compound 1 (0.42 g, 2.00 mmol) in 10 ml of pyridine at 0° (ice-bath). The resultant solution was allowed to stand overnight at 4°. The solvent and the excess acetic anhydride were removed *in vacuo*. The remaining residue was dissolved in $CHCl_3$ (50 ml), washed in a separatory funnel with H_2O (50 ml x 3), saturated $NaHCO_3$ (2 times), and H_2O again (2 times). The $CHCl_3$ solution was clarified with Norit, dried with anhydrous $MgSO_4$, and filtered. The filtrate was then concentrated to a residue which was used immediately without further purification for the next preparation.

The acetate 2 dissolved in 10 ml of pyridine was maintained in a cold-water bath. 4-Chlorophenyl phosphorodichloridate (0.74 g, 3.00 mmol) was added dropwise, followed by 1,2,4-triazole (0.41 g, 6.00 mmol). The mixture was stirred at room temperature for 3 days and then concentrated under reduced pressure (~30°). The resulting residue was dissolved in CH_2Cl_2 (25 ml) and washed with H_2O (25 ml x 2) and 50% $NaHCO_3$ solution (25 ml). The CH_2Cl_2 solution was clarified with Norit, dried ($MgSO_4$), and filtered. The filtrate was evaporated to dryness *in vacuo* to yield a glassy residue (4-triazolylpyrimidinone derivative), which was dissolved in 25 ml of NH_4OH-dioxane (1:3). The mixture was stirred for 5 hr at room temperature in a Wheaton pressure bottle. This solution was then concentrated and the remaining residue was stirred overnight in the pressure bottle at room temperature in saturated methanolic ammonia (25 ml). The solution was then reduced to a small volume *in vacuo* and chromatographed on a silica gel column ($CHCl_3$-MeOH, 3:1, R_f 0.34) to afford 0.17 g (40% based on 1) of product: m.p. 163-165°; UV (0.1 N HCl) λ_{max} 275 nm (ε11,340), λ_{min} 237 nm; UV (0.1 N NaOH) λ_{max} 267 nm (ε7,010), λ_{min} 247 nm; NMR (Me_2SO-d_6) δ3.56 (m, 2H, 5'-H), 4.75 (m, 1H, 4'-H), 4.95 (br s, 1H, 5'-OH, D_2O exchangeable), 5.68 (d, 1H, 5-H), 5.88 (m, 1H, 3'-H, vinyl), 6.33 (m, 1H, 2'-H, vinyl), 6.89 (m, 1H, 1'-H), 7.12-7.19 (br d, 1H, 4-NH_2, D_2O exchangeable), 7.68 (d, 1H, 6-H).

Comparison of the antiviral activities has been made against two retroviruses: Moloney-murine leukemia virus (M-MULV) and HIV. Lin *et al.* (24) have reported previously the antiviral activities of these two analogs against the Moloney-murine leukemia virus. The EC_{50} using the XC-assay described originally by Klement *et al.* (25) and modified by Rowe *et al.* (26) for ddCyd was 4.0 μM and for D4C was 3.7 μM (24). The present report describes the antiviral activities of these two cytidine analogs and AZT against HIV, as evaluated by an assay of reverse transcriptase activity present in solubilized virus obtained from supernatant of virus infected cells, and their effects on infected MT2 cells.

ANTIVIRAL ASSAY IN HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS

Three-day-old mitogen stimulated human peripheral blood mononuclear (PBM) cells (10^6 cells/ml) were infected with HIV (strain LAV) in the presence and absence of various concentrations of 2',3'-dideoxycytidine, D4C and AZT, and 5 days after infection the virus in the supernatant was pelleted and, after disruption, the reverse transcriptase activity 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.* (27). The virus was added to the cultures at the same time as the drugs. The data shown in Fig. 1 clearly indicate that the two compounds were both active with approximate EC_{50} values of 0.005 μM for D4C, 0.011 μM for 2',3'-dideoxycytidine, and about 0.002 μM for AZT, as determined by the Median Effect Method (28).

The effects of the drugs on the growth of uninfected human PBM cells were also established. Mitogen-stimulated PBM cells (*ca.* 2 x 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 6 days using the trypan blue exclusion method. The results suggest that, on day 4 after treatment, both AZT and D4C were toxic at 100 μM to these cells (Fig. 2); at the same concentration, dideoxycytidine was not toxic (data not shown). None of the compounds was toxic at 10- or 100-fold lower concentrations. At the 100-fold lower concentration all three compounds produced almost complete inhibition of the replication of the HIV (Fig. 1).

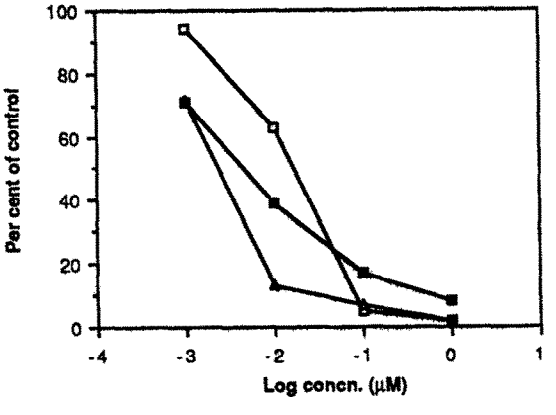


Fig. 1. Effects of 2',3'-dideoxycytidine (□), 2',3'-dideoxycytidin-2'-ene (■), and AZT (△) on the replication of HIV in human PBM cells.

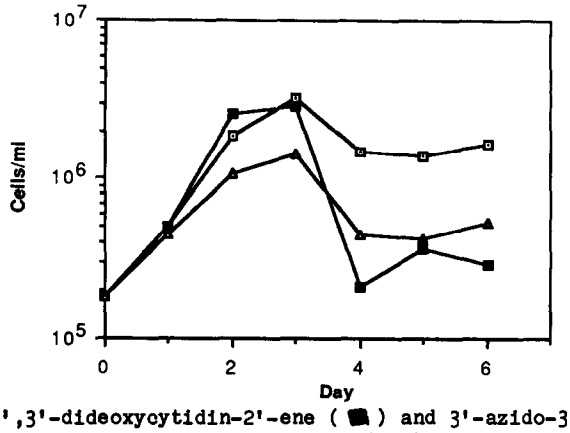


Fig. 2. Effects of 2',3'-dideoxycytidin-2'-ene (■) and 3'-azido-3'-deoxythymidine (△) on the growth of human PBM cells compared to control (⊡).

ANTIVIRAL ASSAY IN HUMAN MT-2 CELLS

The assays in MT-2 cells measured the inhibition of HIV by evaluation of (a) the cytopathic effect (CPE) on HTLV-1 transformed MT-2 cells, (b) the formation of the p18 gag-gene product by use of a specific monoclonal antibody which had been conjugated with Evans blue counterstain, and (c) the viability of the cells using the trypan blue exclusion method. The data are shown in Table 1.

Table 1. Effects of 2',3'-dideoxycytidine, 2',3'-dideoxycytidin-2'-ene and 3'-azido-3'-deoxythymidine on the replication of HIV in MT2 cells

Compound	% Fluorescence+			% Apparent CPE*			% Viable Cells**		
	0.5 μM	1.0 μM	2.0 μM	0.5 μM	1.0 μM	2.0 μM	0.5 μM	1.0 μM	2.0 μM
3'-Azido-3'-deoxythymidine	--	--	1.5±.4	--	--	1±0	--	--	91
2',3'-Dideoxycytidine	4.7±0.3	0.2±0.1	--	40±10	1.0±0.0	--	75	96	--
2',3'-Dideoxy-cytidin-2'-ene	5.6±5.5	1.1±0.1	--	90±0.0	0.5±0.5	--	22	90	--

+ Percent of cells containing viral antigen on day 7 post-infection (day 8 after drug treatment) as demonstrated by immunofluorescence with a monoclonal antibody to p18 antigen. Cultures were in 24-well cell culture plates with 3.3×10^5 cells per well, and 91% of infected control cells fluoresced. Cell counts reflect one field from each duplicate for each drug concentration. The percent is an average of two determinations , and the ± indicates the range of the two values.

* Percent of HIV-infected MT-2 monolayer showing cytopathic effects 7 days after infection (day 8 after drug treatment). The percent is an average of two determinations, and the ± indicates the range of the two values.

** Percent viability of harvested cells on day 7 post-infection (day 8 after drug treatment) from CPE assay, as measured by trypan blue exclusion.

CONCLUSION

The data indicate that 2',3'-dideoxycytidin-2'-ene has inhibitory activity against HIV which is essentially the same as that of 2',3'-dideoxycytidine and AZT when one considers the variation in these assay procedures. The molecular basis for the activity of 2',3'-dideoxycytidin-2'-ene is under study. Depending on the concentration of 3'-azido-3'-deoxythymidine, this compound was either less or more potent than the deoxycytidine analogs in human PBM cells (Fig. 1), and essentially no differences were found in the inhibition of the cytopathic effect produced in HIV-infected MT-2 cells (Table 1). The concentration of AZT required to inhibit the cytopathic effect of HTLV-III_B against helper/inducer T cells was found by Mitsuya et al. (19) to be essentially in the same range as we observed in our system. They found that AZT (1 μM) allows about 40% CPE, whereas we found that 2',3'-dideoxycytidin-2'-ene (1 μM) permitted only 1% CPE (Table 1). Mitsuya and Broder (20) found that 2',3'-dideoxycytidine inhibits the cytopathic effect of HIV against ATH8 cells totally at 0.5 μM and by about one-third at 0.1 μM concentrations. Some variation in the amount of inhibition may be due to differences in strain of virus, the host cell utilized, or the multiplicity of infection.

The potent activity of 2',3'-dideoxycytidin-2'-ene against HIV in fresh and continuous human T cells, and its 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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