Vol. 142, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 15, 1987 Pages 128-134

BOTH 2',3'-DIDEOXYTHYMIDINE AND ITS 2',3'-UNSATURATED DERIVATIVE (2',3'-DIDEOXYTHYMIDINENE) ARE POTENT AND SELECTIVE INHIBITORS OF HUMAN IMMUNODEFICIENCY VIRUS REPLICATION IN VITRO

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Received November 25, 1986

2',3'-Dideoxythymidine (ddThd) and its 2',3'-unsaturated derivative 2',3'-dideoxythymidinene (ddeThd) are potent and selective inhibitors of human immunodeficiency virus (HIV) in vitro. When evaluated for their inhibitory effects on the cytopathogenicity of HIV in MT-4 cells, ddThd and ddeThd completely protected the cells against destruction by the virus at a concentration of 1 μM and 0.04 μM, respectively. In this aspect, ddeThd was about 5 times more potent than 2',3'-dideoxycytidine (ddCyd), one of the most potent and selective anti-HIV compounds now pursued for its therapeutic potential in the treatment of AIDS. ddThd and ddeThd also suppressed HIV antigen expression at 1 μM and 0.04 μM, respectively. Their selectivity indexes, as based on the ratio of the 50 % cytotoxic dose to the 50 % antiviral effective dose, were 120 (ddeThd) and > 625 (ddThd).

Human immunodeficiency virus (HIV), which has recently been designated as the substitute for human T-cell lymphotropic virus type-III/lymphadenopathy virus (HTLV-III/LAV), is the causative agent of acquired immunodeficiency syndrome (AIDS) (1-3). Recent chemotherapeutic approaches toward this systemic and life-threatening disease yielded some promising candidates. Mitsuya et al. (4) reported that 3'-azido-2',3'-dideoxythymidine (N_3ddThd, azidothymidine, AZT) inhibited infectivity and cytopathic effect (CPE) of HIV with minimal cytotoxicity in vitro, and the initial clinical trials with AZT are now in progress (5). Mitsuya and Broder (6) also reported that the 2',3'-dideoxyribosyl derivatives of purines (adenine, guanine, and hypoxanthine) and pyrimidines (thymine and cytosine) significantly suppress HIV CPE in vitro. In particular, 2',3'-dideoxycytidine (ddCyd) was found to be the most potent of the 2',3'-dideoxynucleosides evaluated so far. More recently, Balzarini et al. (7) ascertained that 2',3'-dideoxycytidinene (ddeCyd), which is the 2',3'-unsaturated derivative of ddCyd, is, like ddCyd itself, a potent and selective inhibitor of HIV in vitro.

We have now extended these studies to the 2',3'-unsaturated derivatives of the other 2',3'-dideoxynucleosides, i.e. 2',3'-dideoxyadenosine (ddAdo), 2',3'-dideoxythymidine (ddThd) and 2',3'-dideoxyuridine (ddUrd). Among the newly syn-

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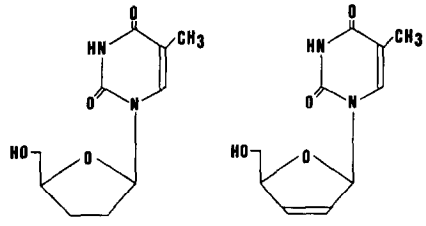

Fig. 1. Structural formulae of 2',3'-dideoxythymidine (ddThd) and 2',3'-dideoxythymidinene (ddeThd).

thesized 2',3'-dideoxyadenosinene (ddeAdo), 2',3'-dideoxyuridinene (ddeUrd), and 2',3'-dideoxythymidinene (ddeThd), the latter proved to be an highly potent anti-HIV agent. It was more potent, though less selective, than its 2',3'-saturated counterpart, ddThd. In their activity as anti-HIV agents, ddThd and ddeThd (Fig. 1) exhibited a selectivity and potency comparable to that of ddCyd. However, ddThd and ddeThd were less potent HIV inhibitors than AZT.

MATERIALS AND METHODS

Compounds. ddCyd was purchased from Pharmacia; ddAdo and ddThd were purchased from Calbiochem-Behring;ddeCyd, ddeThd, ddeAdo and ddeUrd were synthesized in our laboratory. All stock solutions were made in phosphate-buffered saline (PBS) and stored at 4°C until used. Concentrations of the compounds were determined by spectrophotometry.

Virus. HIV was obtained from the culture supernatant of a persistently HIV-infected H9 cell line (H9/HTLV-III$_B$) (8). Virus titer was determined by the $CCID_{50}$ (50 % cell culture infective dose) method and stored at -70°C until used.

Cells. MT-4 cells, which is a human T4 cell line carrying HTLV-I (9), were used throughout all the assays.

Antiviral assays. Antiviral activity of the compounds were based on the inhibition of virus-induced CPE, determined by trypan blue exclusion as previously described (4,6). Briefly, MT-4 cells were adjusted at 5×10^5 cells/ml with RPMI 1640 medium supplemented with 10 % fetal calf serum (FCS) and antibiotics (cell culture medium), and 2 ml portions of the cell suspension were infected with 200 µl of virus stock (titer : $10^{3.8}$ $CCID_{50}$/ml; final titer : 300 $CCID_{50}$/well). Control MT-4 cells were mock-infected with 200 µl of cell culture medium. The cells were incubated at 37°C. After 90 min incubation, 1.8 ml of culture medium was added to each tube. Various dilutions (100 µl) of the test compounds in cell culture medium were prepared previously in flat bottomed 96-well plastic microtiter trays. Then 100 µl of HIV-infected or mock-infected MT-4 cell suspensions were added to the wells, and the cell cultures were incubated at 37°C. After five days, the viability of the cells was examined microscopically in a hematocytometer by the trypan blue exclusion method.

Inhibition of viral antigen expression in HIV-infected MT-4 cells. The inhibitory effects of the compounds on the viral antigen expression in infected MT-4 cells were determined by indirect immunofluorescence (IF) and laser flow cytofluorography using a polyclonal antibody as probe, as previously described (10).

RESULTS

When the 2',3'-dideoxynucleosides (ddAdo, ddThd, ddCyd, and ddUrd) and their 2',3'-unsaturated counterparts (ddeAdo, ddeThd, ddeCyd, and ddeUrd) were compared for their inhibitory effects on the cytopathogenicity of HIV in MT-4 cells, ddeThd proved the most potent in protecting the cells against destruction by the virus. At 0.04 μM, ddeThd completely protected MT-4 cells, whereas the second most potent compound, ddCyd, required a concentration of 0.2 μM to achieve full protection. ddeThd afforded approximately 45 % protection at 0.008 μM, at which concentration ddCyd had no inhibitory effect at all (Fig. 2).

With ddThd full protection against HIV CPE was achieved at a concentration of 1 μM. Similarly, ddeCyd fully protected the cells against HIV at a concentration of 1 μM. The other compounds were much less potent in their anti-HIV

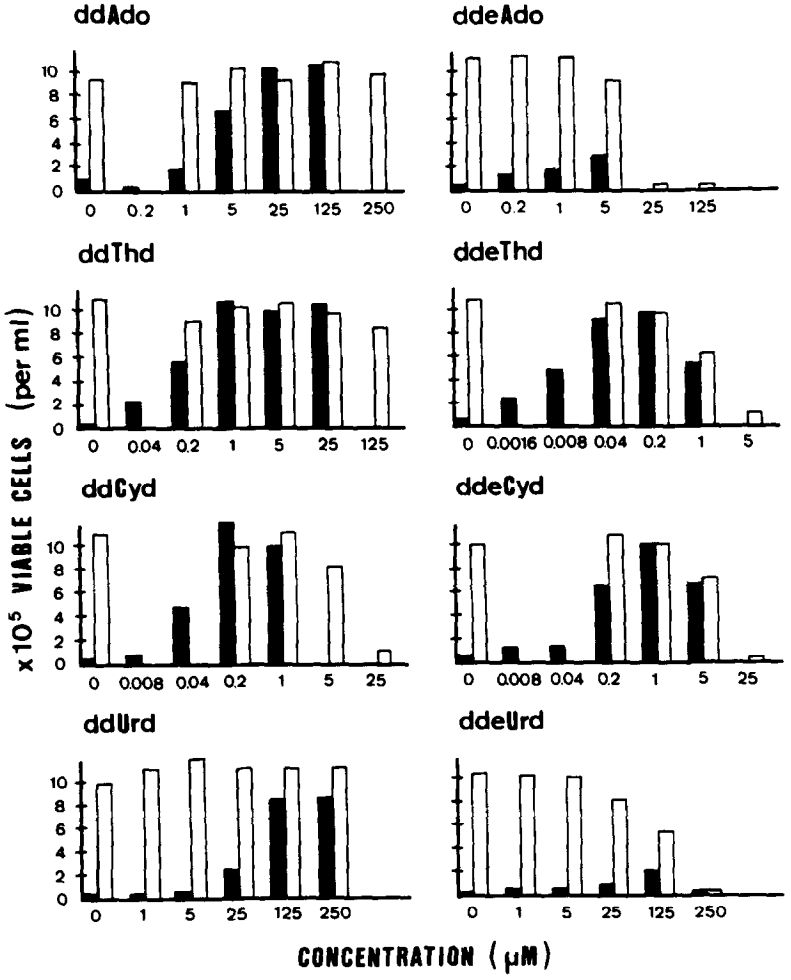

Fig. 2. Inhibition of the cytopathogenicity of HIV in MT-4 cells by 2',3'-dideoxynucleosides and their 2',3'-unsaturated derivatives. Viability of the cells was measured by the trypan blue exclusion method on the 5th day after infection. The infected cells are indicated by solid columns (■) and the mock-infected cells are indicated by open columns (□).

TABLE 1. INHIBITORY EFFECTS OF 2',3'-DIDEOXYNUCLEOSIDES AND THEIR 2',3'-UNSATURATED DERIVATIVES ON THE REPLICATION OF HIV IN MT-4 CELL CULTURES

Compound	ED_{50}[a] (μM)	TD_{50}[b] (μM)	SI[c]
ddAdo	2.5	> 250	> 100
ddeAdo	> 125	9.5	< 0.08
ddThd	0.20	> 125	> 625
ddeThd	0.010	1.2	120
ddCyd	0.046	9.1	198
ddeCyd	0.13	7.9	61
ddUrd	48	> 250	> 5.2
ddeUrd	> 125	27	< 0.22
ddCyd[d]	0.06	37	616
AzddThd (AZT)[d]	0.006	3.5	583

[a] 50 % antiviral effective dose.
[b] 50 % cytotoxic dose.
[c] Selectivity index.
[d] Data taken from ref. 10.

activity, ddAdo achieving full protection only at 25 μM, ddUrd achieving 80 % protection at 125 and 250 μM, and ddeAdo and ddeUrd being virtually devoid of protective activity.

When assayed for cytotoxicity in mock-infected MT-4 cells, ddedThd inhibited cell viability by 45 % at a concentration of 1 μM; whereas ddCyd effected a 25 % reduction in cell viability at a concentration of 5 μM (Fig. 2). The selectivity index (SI) as based on the ratio of the 50 % cytotoxic dose (TD_{50}) to the 50 % antiviral effective dose (ED_{50}), was 120 and 198 for ddeThd and ddCyd, respectively (Table 1).

The higher selectivity index (> 625) was shown by ddThd (Table 1). It is remarkable that this compound was not toxic at concentrations up to 125 μM. This contrasts with ddeThd, ddCyd and ddeCyd which were toxic for the host cells in the concentration range of 1-10 μM. ddeCyd and ddAdo showed SI value of 61 and > 100, respectively. ddUrd had a SI of > 5, whereas the remaining two compounds ddeAdo and ddeUrd were negatively selective in their anti-HIV activity.

When the inhibitory effect of these compounds on viral antigen expression in HIV-infected MT-4 cells were examined, ddeThd achieved complete inhibition of viral antigen expression at a concentration of 0.04 μM (Fig. 3). This concentration was the same as that required for complete protection against HIV cytopathogenicity (Fig. 2). Similarly, ddCyd completely suppressed viral antigen formation at 0.2 μM, a concentration at which it also proved fully protective against viral cytopathogenicity. As a rule, the relative activities of the compounds in inhibiting HIV antigen production correlated closely with their inhibitory effects on HIV cytopathogenicity.

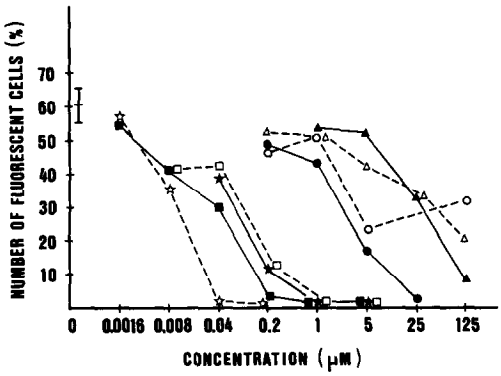

Fig. 3. Inhibition of viral antigen expression in HIV-infected MT-4 cells by 2',3'-dideoxynucleosides and their 2',3'-unsaturated derivatives. Antigen-positive cells were measured by indirect immunofluorescence and laser flow cytofluorography, using polyclonal antibodies from an AIDS patient. The indications of each symbol are as follows : ddAdo (●); ddeAdo (○); ddThd (★); ddeThd (☆); ddCyd (■); ddeCyd (□); ddUrd (▲); ddeUrd (△). The mean percentage of fluorescent cells in absence of compound (positive control) was 60 ± 5 % (—), and that of mock-infected cells (negative control) was 1.0 %.

DISCUSSION

The results presented here indicate that in addition to ddCyd and ddeCyd, which have been previously recognized as potent and selective inhibitors of HIV replication (6,7), ddThd and ddeThd also offer great potential as chemotherapeutic agents for the treatment of AIDS and related retrovirus infections. While ddeThd emerged as the most potent HIV inhibitor among the test compounds, ddThd was the most selective.

As a rule, the 2',3'-unsaturated 2',3'-dideoxynucleosides were more toxic to the uninfected host cells than their saturated counterparts (Table 1). Although ddeThd followed this rule, its selectivity index was only slightly inferior to that of ddCyd when evaluated in parallel (SI : 120 and 198, respectively : see Table 1).

Whereas both ddeThd and ddThd were less potent inhibitors of HIV than AZT, presently the most intensively pursued anti-AIDS drug in clinical trials, the selectivity of ddThd was at least equal if not higher than that of AZT (Table 1).

The mechanism by which ddThd and ddeThd inhibit the replication of HIV remains subject of further study. The 2',3'-dideoxynucleosides and their derivatives, i.e. AZT, are assumed to be targeted at, and to act as chain terminators of, the HIV reverse transcriptase (11). The inhibitory action of ddCyd and ddeCyd against HIV replication is higly dependent on their phosphorylation by deoxycytidine kinase (7). It may be postulated that ddThd and ddeThd are phosphorylated by deoxythymidine kinase, and once converted to their 5'-triphosphate form, interact as chain terminators with the HIV reverse transcriptase.

Mitsuya and Broder (6) reported that ddCyd and ddThd completely inhibited HIV cytopathogenicity at a concentration of 0.5 μM and 200 μM, respectively. In our assays ddCyd and ddThd achieved complete protection against HIV at a concentration of 0.2 μM and 1 μM, respectively (Fig. 2). According to the data of Mitsuya and Broder (6), ddAdo offered full protection against HIV at a concentration of 10 μM, a value which corresponded closely to ours (Fig. 2). In our hands, AZT conferred full protection against HIV at a concentration of 0.02 μM (10), whereas Mitsuya et al. (4) did not obtain full protection with AZT unless the concentration was raised to 1-5 μM. Hence, there appears to be remarkable differences in the sensitivity of HIV to thymidine analogues (i.e. AZT, ddThd) between our own and Mitsuya's studies (4,6). These differences may obviously be related to the assay conditions used, i.e. virus input and choice of the host cells (ATH8 versus MT-4 cells), but as these differences were only observed with thymidine analogues, they may be attributed to differences in the metabolism (i.e. phosphorylation pattern) of the thymidine analogues between ATH8 and MT-4 cells.

The results presented here indicate that ddThd and ddeThd, akin to AZT (4), ddCyd (6), and ddeCyd (7), be further evaluated for their efficacy in the treatment of retrovirus infections, i.e. AIDS.

ACKNOWLEDGEMENTS

HIV (HTLV-III_B) was kindly provided by R.C. Gallo (National Cancer Institute, Bethesda, MD, USA). MT-4 cells were a gift from L. Montagnier (Pasteur Institute, Paris, France). M. Baba is the recipient of a research assistantship from the Onderzoeksfonds of the Katholieke Universiteit Leuven. P. Herdewijn is a Research Associate of the Belgian N.F.W.O. (Nationaal Fonds voor Wetenschappelijk Onderzoek). These investigations were supported in part by the AIDS Basic Research Programme of the European Community and by grants from the Belgian F.G.W.O. (Fonds voor Geneeskundig Wetenschappelijk Onderzoek: Project no. 3.0040.83) and the Belgian G.O.A. (Geconcerteerde Onderzoeksacties: Project no. 85/90-79). We thank Francis Vanlangendonck and Hilde Azijn for excellent technical assistance and Christiane Callebaut for fine editorial help.

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