0026-895X/87/010162-06$00.00/0

MOLECULAR PHARMACOLOGY, 32:162–167

The Anti-HTLV-III (Anti-HIV) and Cytotoxic Activity of 2′,3′-Didehydro-2′,3′-dideoxyribonucleosides: A Comparison with Their Parental 2′,3′-Dideoxyribonucleosides

JAN BALZARINI,*§ GIL-JONG KANG,‡ MAHA DALAL,‡ PIET HERDEWIJN,§ ERIK DE CLERCQ,§ SAMUEL BRODER,* and DAVID G. JOHNS‡

*Clinical Oncology Program and ‡Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892 and §Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium

Received January 21, 1987; Accepted April 1, 1987

SUMMARY

A series of 2′,3′-didehydro-2′,3′-dideoxyribonucleosides (ddeNs) [i.e., 2′,3′-dideoxythymidinene (ddeThd), 2′,3′-dideoxyuridinene (ddeUrd), 2′,3′-dideoxycytidinene (ddeCyd), and 2′,3′-dideoxyadenosinene (ddeAdo)] has been synthesized and the individual members compared in terms of their *in vitro* antiviral, antimetabolic, and cytostatic properties to their 2′,3′-saturated counterparts (ddNs) (i.e., ddThd, ddUrd, ddCyd and ddAdo). All ddeNs except ddeUrd are potent and/or selective inhibitors of human immunodeficiency virus (HIV) *in vitro*, ddeCyd being the most potent (MIC_{50}, 0.30 μM). The inhibitory effect of ddeCyd on ATH8 cell proliferation and HIV-induced cytopathogenicity is comparable to that of ddCyd. ddeThd is a more potent anti-HIV agent than ddThd (MIC_{50}, 3.4 μM and 84 μM, respectively), but also more cytostatic (ID_{50}, 172 μM and >2000 μM, respectively). However, its *in vitro* chemotherapeutic index is higher than that of 3′-azido-2′,3′-dideoxythymidine, a drug which has recently proven effective in the treatment of acquired immunodeficiency syndrome. ddeAdo has a weaker anti-HIV and a stronger cytostatic effect than ddAdo. Neither ddeUrd nor ddUrd shows significant anti-retroviral activity at 500 μM. In contrast to their anti-retroviral activity, both ddNs and ddeNs lack any appreciable inhibitory activity against a series of non-oncogenic RNA and DNA viruses, pointing to their selectivity as anti-retroviral agents. All ddeNs show a progressive loss of anti-retroviral effect upon prolonged incubation with virus-infected cells. This phenomenon is most likely due to the chemical instability of these compounds, and not to a preferential enzymatic phosphorolytic cleavage of the ddeNs. Evidence is presented that ddeCyd and ddCyd, and ddeThd and ddThd are phosphorylated by cellular dCyd kinase and dThd kinase, respectively. However, the K_i values as alternate substrate inhibitors for their respective kinases are high (>500 μM), indicating poor substrate activity and, thus, poor anabolism in ATH8 cells.

HTLV-III/LAV, also designated HIV, has been identified as the etiologic agent of AIDS (1–3). There is considerable current interest in the development of agents that prove effective in the treatment of this disease.

Recently, Mitsuya *et al.* (4) reported that AZT is a potent inhibitor of HIV replication in human lymphocyte cultures and protects the cells against the cytopathic effects of the virus at a concentration of 1–5 μM. Although the mechanism of action of this compound has not yet been fully elucidated, it has been speculated that inhibition of the virus-encoded DNA polymerase (reverse transcriptase) may account, at least in part, for its selectivity. Indeed, Furman *et al.* (5) have demonstrated that the 5′-triphosphate of AZT selectively inhibits HIV reverse transcriptase at a concentration more than 100-fold lower than that at which it inhibits DNA polymerase α. Moreover, should AZT itself be incorporated, the absence of a hydroxyl group at the 3′-position of the sugar moiety would result in chain termination. This characteristic also holds for the 2′,3′-dideoxyribonucleotide analogues of the natural 2′-deoxyribonucleoside triphosphates: Atkinson *et al.* (6) demonstrated that 2′,3′-dideoxythymidine-5′-triphosphate (ddTTP) and also 2′,3′-didehydro-2′,3′-dideoxythymidine-5′-triphosphate (ddeTTP) cause chain termination after incorporation into DNA.

Mitsuya and Broder (7) demonstrated that every purine (adenosine, guanosine, and inosine) and pyrimidine (cytidine

These investigations were supported in part by grants from the Belgian Fonds voor Geneeskundig Wetenschappelijk Onderzoek (Project No. 3.0040.83) and the Belgian Geconcerteerde Onderzoeksacties (Project No. 85/90-79). P. H. is a research associate of the Belgian Nationaal Fonds voor Wetenschappelijk Onderzoek.

ABBREVIATIONS: HTLV-III/LAV, human T-cell lymphotropic virus type III/lymphadenopathy-associated virus; HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; AZT, 3′-azido-2′,3′-dideoxythymidine (BW A509U, azidothymidine); L1210/0, murine leukemia L1210 cells; L1210/BdUrd, deoxythymidine kinase-deficient L1210 cells; L1210/araC, deoxycytidine kinase-deficient L1210 cells; ddeN, 2′,3′-didehydro-2′,3′-dideoxyribonucleoside; ddN, 2′,3′-dideoxyribonucleoside; araC, arabinofuranosylcytosine.

and thymidine) nucleoside containing the 2′,3′-dideoxyribose configuration, when evaluated against the infectivity and cytopathic effect of HIV *in vitro*, significantly suppressed the virus, ddCyd being the most potent of the series (total antiviral protection at 0.5–1.0 μM). However, the olefinic ddNs have never been subjected to extensive antiviral examination, although the synthesis of these compounds was reported some years ago. The introduction of a 2′,3′-double bond into the ribose moiety of ddUrd, ddCyd and ddThd was originally described by Horwitz *et al.* (8–10), while Robins and Robins (11) and McCarthy *et al.* (12) reported the synthesis of ddAdo and its 2′,3′-unsaturated counterpart (ddeAdo).

In search of new potential inhibitors of HIV, we have now resynthesized three 2′,3′-unsaturated pyrimidine dideoxynucleoside analogues (ddeThd, ddeUrd and ddeCyd) and one 2′,3′-unsaturated purine dideoxynucleoside analogue (ddeAdo), and compared their *in vitro* antiviral, antimetabolic, and cytostatic effects to those of the corresponding saturated ddNs, ddThd, ddUrd, ddCyd and ddAdo. A preliminary account of some of our results with one member of the pyrimidine series (ddeCyd) has recently appeared (13).

Materials and Methods

Compounds. ddCyd, ddThd, and ddAdo were obtained from Pharmacia-PL-Biochemicals (Piscataway, NJ). Previous published methods were used to synthesize the following sugar-modified nucleosides: ddeCyd (8), ddUrd (10), ddeUrd (10), ddeAdo (12), and ddeThd (14). The other reagents used were of the highest quality available.

Cells. The origin, cultivation, and characterization of murine leukemia L1210 cells, designated L1210/0; deoxythymidine kinase-deficient L1210 cells, designated L1210/BdUrd; deoxycytidine kinase-deficient L1210 cells, designated L1210/araC; murine mammary carcinoma FM3A cells; human B-lymphoblast Raji cells; human T-lymphoblast Molt/4F cells; and human T4-lymphocyte ATH8 cells have previously been described (15–18).

Radiochemicals. [2-^{14}C]dThd (specific radioactivity 50–60 mCi/mmol) and [5-^{3}H]dCyd (specific radioactivity 22 Ci/mmol) were obtained from Amersham International Limited (Amersham, England). [5,6-^{3}H]ddCyd (specific radioactivity 6 and 5.5 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA).

Cytostatic assays. Cytostatic effects of the compounds were assessed by measuring the inhibition of cell proliferation. The experimental procedures have been described previously (16–19). Briefly, L1210/0, L1210/BdUrd, L1210/araC, FM3A, Raji, and Molt/4F cells were suspended in growth medium and added to a microplate well at a density of 5×10^4 cells/well in the presence of varying concentrations of the test compounds. The cells were then allowed to proliferate for 48 hr at 37°. At the end of the incubation period, the cells were counted in a Coulter counter. The ID_{50} was defined as the concentration of compound that reduced the number of cells by 50%.

Viruses. HTLV-III$_B$ (designated HIV) was derived from a pool of American patients with AIDS. Approximately 6×10^{10} virus particles/ml were obtained from the culture supernatant of HIV-producing H9 cells as previously described (4).

The origin of the other viruses was as follows: herpes simplex virus type 1 (strain KOS) and type 2 (strain G), see Ref. 20; vaccinia virus, vesicular stomatitis virus, Sindbis virus, Coxsackie virus type B4, and polio virus type 1, see Ref. 21; reovirus type 1 (ATCC VR-230), parainfluenza virus type 3 (ATCC VR-93), and Semliki forest virus were obtained from the American Type Culture Collection (Rockville, MD).

Antiviral assays. The antiviral assays, other than HTLV-III/LAV, were based on an inhibition of virus-induced cytopathogenicity in either Hela cell, Vero cell, or primary rabbit kidney cell cultures, following previously established procedures (20). Briefly, confluent cell cultures in microtiter trays were inoculated with 100 $CCID_{50}$ of virus, 1 $CCID_{50}$ being the virus dose required to infect 50% of the cell cultures. After 1 hr of virus adsorption, residual virus was removed and the cell cultures were incubated in the presence of varying concentrations (400, 200, 100, ... μg/ml) of the test compounds. Viral cytopathogenicity was recorded as soon as it reached completion in the control virus-infected cell cultures.

Anti-HIV assay. Human T-lymphocyte ATH8 cells were pretreated with polybrene at 2 μg/ml for 30 min at 37°. Cells were then pelleted, suspended in fresh RPMI-1640 culture medium containing 13% fetal calf serum, 11% interleukin-2 (v/v), 50 μM β-mercaptoethanol, 4 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin, and infected with 2×10^3 virions/cell for 60–90 min at 37°. This virus concentration represents 400 times the minimum dose required to induce cytopathogenicity in ATH8 cells and, thus, represents a very high multiplicity of infection (19). After infection, cells were reconstituted in culture medium and seeded in culture tubes at 2 ml/tube in the presence or absence (controls) of the test compound. After incubation for 6–7 or 10 days at 37°, the number of viable cells was counted and compared to controls (noninfected cells which were incubated with the same concentrations of the compounds as the virus-infected cells) (19).

Enzyme assays. dCyd kinase and dThd kinase were prepared from exponentially growing Molt/4F cells, which were washed twice with phosphate-buffered saline at 4° and homogenized with a Polytron homogenizer (Brinkmann Instruments, Rexdale, Ontario, Canada) for dCyd kinase or sonicated three times for 10 sec for dThd kinase preparation.

The suspensions were then clarified by centrifugation at 25,000–27,000 × *g* for 30 min. The 30–60% $(NH_4)_2SO_4$ precipitate of the resulting supernatant was resuspended in buffer containing 10 mM Tris-HCl, pH 7.5, and 5 mM β-mercaptoethanol, dialyzed against the same buffer, and used for the dCyd kinase assays. The 30–70% $(NH_4)_2SO_4$ precipitate of the supernatant was resuspended in buffer containing 10 mM potassium phosphate, pH 7.5, 10 mM β-mercaptoethanol and 0.1 M KCl, dialyzed against the same buffer, and used for the dThd kinase assays. The dCyd kinase assays were carried out by a radiometric method as described by Ives and Wang (22); the procedure followed for the dThd kinase assays was described previously (23). In these experiments, [5-^{3}H]dCyd and [2-^{14}C]dThd served as the radiolabeled substrates. The apparent K_m and K_i values were derived from Lineweaver-Burk plots, using a linear regression analysis program.

A continuous spectrophotometric assay was used with purified bacterial dThd phosphorylase (Sigma Chemical Co., St. Louis, MO) using a Beckman spectrophotometer type DB-G. The change in absorbance during the enzyme reaction was continuously monitored at 25° at the wavelength where the difference between the nucleoside and its free base was maximal ($\lambda\Delta A_{max}$): dUrd (270 nm), ddUrd (275 nm), ddeUrd (270 nm), dThd (275 nm), ddThd (280 nm), ddeThd (272 nm), dCyd (276 nm), ddCyd (275 nm), ddeCyd (307 nm).

The reaction mixtures for all of the assays in Table 4 consisted of enzyme and nonsaturating concentrations of nucleoside (20, 25, 30, 40, and 50 μM) in 0.1 M sodium phosphate buffer, pH 7.0. The initial velocity of phosphorolysis has been expressed as nmol/min/enzyme unit at 25 μM test compound and increased linearly within the concentration range of 20–50 μM for all of the compounds tested. Saturating concentrations of dUrd, dThd, and the ddNs have not been determined.

Results

The ddeNs (i.e., ddeThd, ddeUrd, ddeCyd and ddeAdo) and their 2′,3′-saturated counterparts (ddNs) were first compared for their inhibitory effects on HIV-induced cytopathogenicity in ATH8 cells (Table 1). When recorded at day 7 post-infection, there were marked differences in the inhibitory effects of the ddeNs on HIV. With a MIC_{50} of 0.30 μM, ddeCyd was the most potent, and with a MIC_{50} of > 100 μM, ddUrd was the least

TABLE 1

Anti-HIV activity and cytotoxic effects of ddeNs and their 2′,3′-saturated counterparts

Compound	Recorded at day 6–7 after virus inoculation			Recorded at day 10 after virus inoculation		
	MIC_{50}[a]	ID_{50}[b]	ID_{50}/MIC_{50}[c]	MIC_{50}	ID_{50}	ID_{50}/MIC_{50}
	μM			μM		
ddeCyd	0.3	30	100	2.0	35	17.5
ddCyd	0.2	35	175	0.2	35	175
ddeUrd	>100	107	>1.1	>100	>100	
ddUrd	>500	>500		>500	>500	
ddeThd	4.1	110	27	10	100	10
ddThd	101	>2000	>24	220	>2000	>9.2
ddeAdo	40	52	1.3	>100	75	>0.7
ddAdo	2.7	>620	>229	3	>500	>166
AZT	2.4	40	16.6			

[a] Concentration required to reduce virus-induced cytopathogenicity by 50%.
[b] Concentration required to reduce ATH8 cell viability by 50% (compared to untreated control).
[c] The ratio ID_{50}/MIC_{50} represents the *in vitro* "therapeutic index." However, it should be noted that MIC_{50} values will vary with the original multiplicity of viral infection, while the ID_{50} values will not. Thus, a higher multiplicity of infection will give a lower ID_{50}/MIC_{50} ratio and vice versa. However, the relative ranking of the therapeutic indices within a series of compounds will remain the same.

effective. ddeAdo and ddeThd showed MIC_{50} values that were intermediate between these extremes. ddeCyd was almost as effective as ddCyd in inhibiting the cytopathogenicity of HIV in ATH8 cells, and also proved equally as cytostatic as ddCyd to the human lymphocytes. On the other hand, ddeThd protected ATH8 cells against HIV to a considerably greater extent than ddThd (~25-fold), but was also more cytostatic (~20-fold). Its antiviral activity proved to be similar to that of AZT, an anti-HIV drug recently found to be effective in AIDS patients (Fig. 1). In contrast, ddeAdo was not only less effective than its saturated counterpart (ddAdo) as an anti-retroviral agent, but also more cytostatic than ddAdo for ATH8 cells. Thus, among the ddeNs, only ddeCyd and ddeThd had *in vitro* chemotherapeutic indices close to those of ddCyd and ddThd, respectively. The other ddeNs tested showed lower chemotherapeutic indices than their parental ddNs. However, upon longer incubation with the HIV-infected ATH8 cells, all ddeNs including ddeCyd and ddeThd partially lost their anti-retroviral activity (Table 1), whereas their cytostatic effects did not markedly change.

When both ddeNs and ddNs were examined against a number of non-oncogenic viruses including the DNA viruses herpes simplex virus type 1, herpes simplex virus type 2, and vaccinia virus, and the RNA viruses vesicular stomatitis virus, Sindbis virus, Coxsackie virus type B, polio virus type 1, reovirus type 1, Semliki forest virus, and parainfluenzavirus type 3, none of the ddeNs and ddNs tested exhibited any marked antiviral activity, and both series of compounds proved 50 to > 1000-fold less effective in inhibiting the virus-induced cytopathogenic effect compared to their anti-retroviral activity.

Cytostatic effects of ddeNs and ddNs. We next examined the cytostatic effects of the ddeNs and their 2′,3′-saturated congeners against several murine (leukemia L1210/0, L1210/BdUrd, L1210/araC, FM3A) and human (B-lymphoblast Raji, T-lymphoblast Molt/4F, and T-lymphocyte ATH8) cell lines. ddeUrd, ddeThd, and ddeAdo were significantly more cytostatic against murine and human cell lines than were their corresponding saturated counterparts (Table 2). No specific inhibitory potencies of the compounds were observed for the proliferation of murine and human cell lines except for ddeThd, which was considerably (>100-fold) more cytostatic against L1210/0 cells than against the other murine and human cell lines evaluated, and ddeCyd and ddCyd, which proved much more inhibitory to human lymphoid cell proliferation (i.e., Raji, Molt/4F, and ATH8; ID_{50}, 12–35 μM) than to murine (i.e., L1210, FM3A; ID_{50} > 160 μM) or to human non-lymphoid (i.e.,

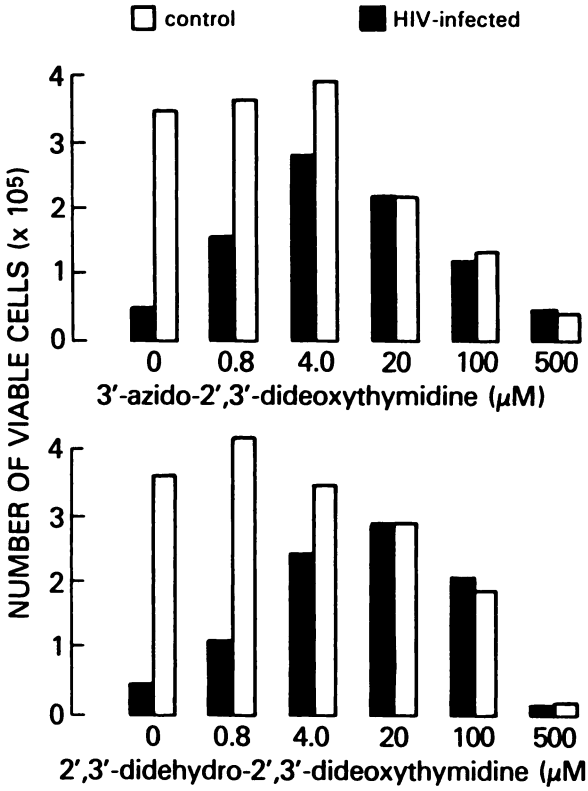

Fig. 1. Inhibition of the cytopathic effect of HIV by AZT and ddeThd against ATH8 cells. ATH8 cells (2×10^5) were preexposed to polybrene, exposed to HIV (2000 virus particles/cell), and cultured in the presence or absence of compound. On day 7 post-infection, total viable cells were counted by trypan blue dye exclusion. □, cell cultures treated with compound only; ■, cell cultures treated with compound after infection with the virus.

TABLE 2

Cytostatic activity of ddeNs and their 2′,3′-saturated counterparts against a series of murine and human cell lines

Cell	ID_{50} (μM)[a]							
	ddCyd	ddeCyd	ddUrd	ddeUrd	ddThd	ddeThd	ddAdo	ddeAdo
L1210/0[b]	320	196	>2000	318	>2000	21	702	178
L1210/BdUrd[c]	298	167	>2000	648	>2000	516	745	156
L1210/araC[d]	>2000	1134	>2000	188	>2000	340	825	123
FM3A[e]	>2000	1158	>2000	413	>2000	504	855	234
Raji[f]	12	13	>2000	223	>2000	668	1208	168
Molt/4F[g]	17	20	>2000	258	>2000	908	968	234
ATH8[h]	35	30	>2000	107	>2000	110	620	52

[a] ID_{50}, dose required to inhibit cell proliferation by 50%. Data represent the average of at least three separate experiments. For ATH8 cells, cytotoxic doses are given, i.e., the concentration required to reduce cell viability by 50%. Results were recorded at day 6–7 after seeding of the cells.
[b] Murine leukemia cells.
[c] L1210 cells deficient in dThd kinase.
[d] L1210 cells deficient in dCyd kinase.
[e] Murine mammary carcinoma cells.
[f] Human B-lymphoblasts.
[g] Human T-lymphoblasts.
[h] Human T4-lymphocytes.

adenosquamous carcinoma NCI-H125, bronchioloalveolar carcinoma NCI-H322 and NCI-H358, and large cell carcinoma NCI-H460; $ID_{50} > 100$ μM) cell proliferation (Table 2 and data not shown). It is worthwhile noting that ddeCyd and ddCyd were much less cytostatic against the L1210/araC cell line than against the parental L1210/0 cells.

When the effect of the addition of natural purine (i.e., Ado, dAdo) and pyrimidine (i.e., dThd, dUrd, dCyd, Urd, Cyd) nucleosides on the cytostatic action of the ddeNs and ddNs was evaluated, dCyd and Cyd dramatically reversed the inhibitory effects of both ddeCyd and ddCyd on Molt/4F cell proliferation (30 to > 100-fold) (Table 3). The effects of other ddeNs and ddNs were not reversed by any of the natural nucleosides tested.

Effect of ddCyd, ddeCyd, ddThd, and ddeThd on partially purified Molt/4F dCyd kinase and dThd kinase. Both ddeCyd and ddCyd were evaluated for their inhibitory effect on partially purified dCyd kinase, derived from human Molt/4F cells. The K_m value of the enzyme for its physiological substrate dCyd was 2.8 μM. ddCyd inhibited dCyd kinase only at high concentrations (K_i, 1.0 mM), whereas ddeCyd exerted an even lower inhibitory effect on the enzyme (K_i, 4.6 mM). Both compounds showed competitive inhibition of dCyd kinase with respect to dCyd within a concentration range of 2–10 μM dCyd. When evaluated for its substrate activity against dCyd kinase, ddCyd showed a K_m value of 201 μM, a 50- to 60-fold higher value than the K_m of dCyd for the enzyme (Fig. 2). The V_{max} for ddCyd phosphorylation was 0.3 times the V_{max} for dCyd conversion (Fig. 2).

ddThd and ddeThd were also examined for their inhibitory effects on Molt/4F dThd kinase. The K_m value of the physiological substrate dThd for the enzyme was 4–5 μM. Both analogues were found to be weakly inhibitory against the enzyme with respect to dThd phosphorylation. They showed comparable K_i values (~580 μM) and behaved as competitive inhibitors for dThd kinase within a concentration range of 5–50 μM dThd.

Nucleoside phosphorolysis. In comparison to the pattern of degradation shown for dUrd and dThd, the 2′,3′-dideoxyribose and the 2′,3′-didehydro-2′,3′-dideoxyribose configuration in the nucleoside substantially decreased the velocity of phosphorolysis by purified bacterial dThd phosphorylase (200- to 1000-fold) (Table 4). Both ddeUrd and ddeThd showed a slightly increased initial velocity for phosphorolytic cleavage compared to their 2′,3′-saturated counterparts. dCyd, ddCyd, and ddeCyd were not subject to phosphorolytic cleavage by the bacterial dThd phosphorylase.

TABLE 3

Effect of natural nucleosides on the cytostatic activity of ddeNs and their 2′,3′-saturated counterparts against human T-lymphoblasts (Molt/4F)

Upon addition of following nucleoside[a]	ID_{50} (μM)[b]							
	ddCyd	ddeCyd	ddUrd	ddeUrd	ddThd	ddeThd	ddAdo	ddeAdo
(Control)	17	20	>2000	258	>2000	908	968	234
dThd (800 μM)	28	29	>2000	197	>2000	672	744	200
dUrd (550 μM)	30	39	>2000	258	>2000	852	994	218
dCyd (2200 μM)	>2000	1440	>2000	237	>2000	616	1070	210
Urd (510 μM)	13	23	>2000	258	>2000	552	1010	222
Cyd (2060 μM)	620	1100	>2000	223	>2000	564	746	198
dAdo (38 μM)							982	236
Ado (470 μM)							974	224

[a] The nucleosides were added at subtoxic concentrations.
[b] ID_{50}, dose required to inhibit cell proliferation by 50%. Data represent the average of at least three separate experiments.

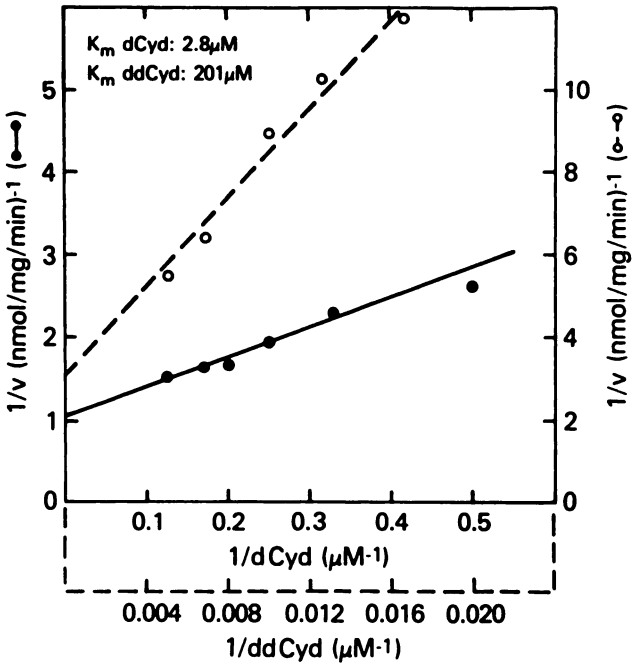

Fig. 2. Double reciprocal plots for substrate activity of dCyd (●—●) and ddCyd (○- - -○) for Molt/4F dCyd kinase. The plotted data are the mean values for at least three separate experiments.

TABLE 4

Initial velocities for phosphorolysis of 2′-deoxyribonucleosides, ddNs, and ddeNs by dThd phosphorylase

Compound	Initial velocity of bacterial dThd phosphorylase[a]
	nmol/min/enzyme unit
dThd	61.2
ddThd	0.043
ddeThd	0.312
dUrd	101
ddUrd	0.080
ddeUrd	0.112
dCyd	ND[b]
ddCyd	ND
ddeCyd	ND

[a] Initial velocity was determined at a substrate concentration of 25 μM.
[b] No detectable phosphorolysis.

Nonenzymatic breakdown of ddeNs and ddNs. Freshly prepared solutions of the ddeNs and ddNs in 0.1 M Tris-HCl buffer at pH 7.0 were incubated for varying time periods at 37°. Time-dependent spontaneous degradation of all ddeNs to their corresponding free bases was observed by high performance liquid chromatographic analysis of the samples taken at different time intervals. Breakdown of ddeAdo was most extensive (50% hydrolysis in less than 1 day), followed by ddeUrd, ddeThd, and ddeCyd (50% hydrolysis after ~3 days) (Fig. 3). None of the corresponding 2′,3′-saturated ddNs showed any high performance liquid chromatography-detectable breakdown after 3 days of incubation (Fig. 3).

Discussion

None of the ddNs or their 2′,3′-unsaturated congeners showed any inhibitory effects against the cytopathogenicity of a number of non-oncogenic DNA and RNA viruses. In contrast, most of the compounds evaluated inhibited the HIV-induced

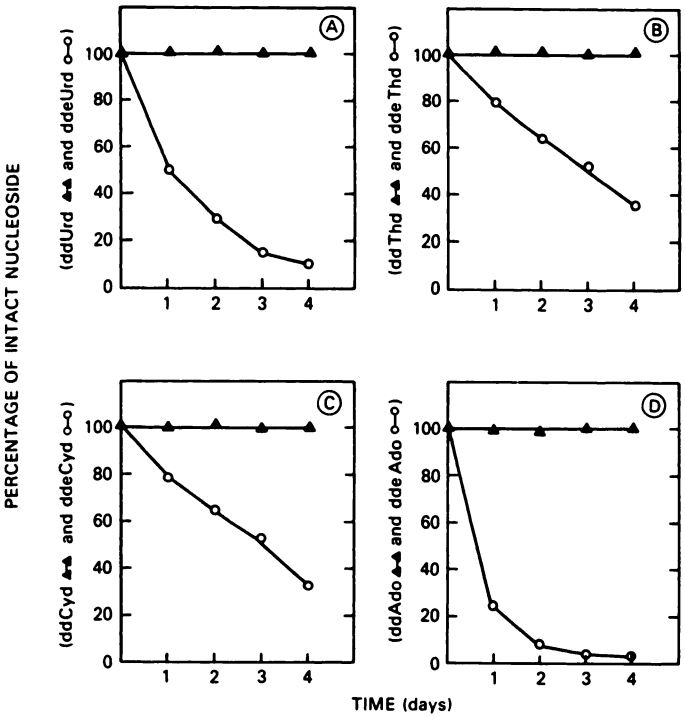

Fig. 3. Spontaneous degradation of 1 mM ddeNs and ddNs at 37° in 0.1 M Tris-HCl, pH 7.0, as a function of time. A: ddUrd (▲) and ddeUrd (○), B: ddThd (▲) and ddeThd (○), C: ddCyd (▲) and ddeCyd (○); D: ddAdo (▲) and ddeAdo (○).

TABLE 5

Comparative kinetic constants of dCyd kinase for ddCyd and araC

	ddCyd	araC[a]
	Molt/4F[b]	Calf thymus[b]
K_m (μM)	201	41
V_{max} (pmol/mg/min)	640	1020
K_i (μM)	997	350
K_i/K_m for dCyd	319	70
K_i/K_m for ddCyd or araC	6.2	8.6

[a] Data for araC are from Durham and Ives (27).
[b] K_m values of dCyd for Molt/4F and calf thymus dCyd kinase are 2.8 μM and 5.3 μM, respectively.

cytopathogenic effect in ATH8 cells, pointing to the selectivity of the ddNs and ddeNs as anti-retroviral agents. It would appear, therefore, that these compounds inhibit a crucial step during the life cycle of the virus which is unique with respect to retroviruses. Most likely, the target for the inhibitory effect of the compounds is the transcription of viral RNA to DNA, catalyzed by the virus-encoded DNA polymerase (reverse transcriptase). Indeed, it has been reported that the 5′-triphosphate derivative of 2′,3′-ddThd inhibits murine and avian oncoviral reverse transcriptase to a more than 100-fold greater extent than it inhibits cellular DNA polymerase α (24). Moreover, Furman *et al.* (5) reported a similar observation for AZT 5′-triphosphate and suggested that the specificity of AZT as an anti-HIV agent may be ascribed, at least in part, to its potent inhibition of HIV reverse transcriptase (5).

Concerning the anti-retroviral effects of the olefinic ddNs, ddeCyd was almost as effective as ddCyd in protecting ATH8 cells against the cytopathogenicity of HIV when recorded at the sixth day after infection. Both compounds are also equally effective in inhibiting antigen expression of HIV in human lymphocyte MT-4 cells (13). Moreover, the cytostatic effects of ddeCyd against human ATH8, Molt/4F, and Raji cells were also comparable to those of ddCyd. Therefore, ddeCyd had an *in vitro* chemotherapeutic index (ratio ID_{50}/MIC_{50}) close to that of ddCyd and, thus, might be considered as a potential anti-retroviral drug worthy of evaluation *in vivo*. The ratio ID_{50}/MIC_{50} of ddCyd and ddeCyd proved to be of the same order of magnitude as that of ddAdo, but higher than those of all other ddeN and ddN analogues tested, including ddeAdo, ddeThd, ddThd, ddeUrd, and ddUrd.

It is unclear why ddeThd shows a 25-fold increase in anti-retroviral activity compared to ddThd, with a parallel increase in cytostatic potential. It is unlikely that higher intracellular levels of ddeTTP than of ddTTP are formed, since the identical K_i values of ddeThd and ddThd for dThd kinase suggest that both drugs have similar susceptibility to phosphorylation, at least to their 5′-monophosphate metabolites. However, Atkinson *et al.* (6) reported that the ability of ddeTTP to inhibit *E. coli* DNA polymerase with poly d(A-T) as the template was 4-fold greater than that of ddTTP under similar experimental conditions. Thus, the increased antiviral and cytostatic effect of ddeThd over ddThd might be explained by differential effects at the cellular DNA polymerase and/or reverse transcriptase level. Moreover, when we compared the antiviral, cytostatic, and cytotoxic effects of ddeThd with AZT, both compounds proved equally effective in protecting ATH8 cells against the cytopathogenicity of HIV, but ddeThd had a higher *in vitro* chemotherapeutic index, i.e., it was less cytostatic and cytotoxic against ATH8 cells than AZT (Fig. 1, Table 1). Since AZT is a drug which has proven effective in AIDS patients, further evaluation of ddeThd, including examination of some of its *in vivo* pharmacological properties would appear warranted.

It remains puzzling why ddCyd is at least 500-fold more potent than ddThd as an anti-retroviral agent. Indeed, we demonstrated that ddCyd and ddThd, and also their 2′,3′-unsaturated derivatives ddeCyd and ddeThd, showed comparable (poor) substrate activities for their phosphorylating enzymes dCyd kinase and dThd kinase, respectively. However, differences in phosphorylation of the 5′-monophosphate derivatives to their respective 5′-triphosphates, unequal incorporation rates into retroviral DNA, and/or different properties of ddN- or ddeN-containing DNA may be responsible for the striking difference in the *in vitro* anti-HIV effect of the two compounds.

The decrease in antiviral potency of all ddeNs upon prolonged incubation with HIV-infected ATH8 cells is not related to a higher susceptibility of these compounds to enzymatic hydrolysis since we found that ddThd, ddeThd, ddCyd, ddeCyd, ddUrd, and ddeUrd were far more resistant to phosphorolytic cleavage by dThd phosphorylase than dThd and dUrd, and no striking differences were noted between the ddNs and the ddeNs. Most likely, as we recently reported for ddeCyd (13), the decrease in protective effects of the ddeNs against HIV-infected cells as a function of the incubation time is due to the chemical instability of this class of compounds, either before or after incorporation into DNA. This explanation corroborates the observation of Sanger et al. (25), that the 5′-triphosphate derivative of ddeThd was much less stable than its corresponding ddThd derivative when utilized in the DNA sequencing reaction. This property might be advantageous *in vivo*, since it would permit a more rapid clearance of the ddeNs compared to the corresponding ddNs if toxicity should occur. However, the

fact that ddThd also loses its antiviral potency upon longer incubation times with HIV-infected cells, but seems not to be hydrolyzed spontaneously under our experimental conditions, points to a more complex explanation of this phenomenon.

The observations that (i) the anti-retroviral and cytostatic effects of ddeCyd and ddCyd against human Molt/4F cells are significantly reversed by dCyd (Ref. 19 and Table 3), (ii) ddeCyd and ddCyd are considerably less cytostatic against a dCyd kinase-deficient cell line compared to the parental cells (Table 2), and (iii) phosphorylated metabolites of ddCyd could not be detected in a dCyd kinase-deficient cell line (26) point to dCyd kinase as the activating enzyme for both ddeCyd and ddCyd. Indeed, partially purified dCyd kinase from Molt/4F cells phosphorylates dCyd and ddCyd, with dCyd being the kinetically preferred substrate. ddeCyd (and also ddCyd) competitively inhibit the phosphorylation of dCyd within a substrate range of 2–10 μM. The kinetic constants for dCyd and ddCyd indicate that, in the presence of both substrates, dCyd will be preferentially utilized. There is a striking analogy between the kinetic behavior of ddCyd and araC toward dCyd kinase (Table 5). The K_m and K_i values of ddCyd for dCyd kinase are 3- to 5-fold higher than those of araC, and both araC and ddCyd showed competitive inhibition with respect to dCyd within a concentration range of 2–10 μM. These data suggest that ddCyd is an even poorer substrate for dCyd kinase than is araC. Despite this, however, intracellular levels of ddCTP can be reached in a number of human cell lines, including ATH8 cells, that are high enough to account for effective antiviral activity (26). In this respect, ddeCyd is probably not more efficiently phosphorylated than is ddCyd, and one may also expect that no higher intracellular ddeCTP levels will be reached under the same conditions as for ddCyd.

In conclusion, ddeNs are a new class of anti-HIV compounds endowed with selective and potent anti-retroviral activity. Of the 2′,3′-unsaturated compounds examined, ddeCyd and ddeThd appear sufficiently promising to warrant further pharmacological studies.

Acknowledgments

HTLV-III was kindly provided by Dr. R. C. Gallo, National Cancer Institute, National Institutes of Health, Bethesda, MD. We thank Miette Stuyck and Lizette Van Berckelaer for excellent technical help, and Beth Singer, Yetta Buckberg, and Tanya Prather for fine editorial assistance.

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Send reprint requests to: Dr. David G. Johns, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, 37/5B22, National Institutes of Health, Bethesda, MD 20892.