Vol. 145, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1987 Pages 269-276

THE 2',3'-DIDEOXYRIBOSIDE OF 2,6-DIAMINOPURINE SELECTIVELY INHIBITS HUMAN IMMUNODEFICIENCY VIRUS (HIV) REPLICATION IN VITRO

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Received April 9, 1987

SUMMARY : The 2',3'-dideoxyriboside of 2,6-diaminopurine(ddDAPR) is, like 2',3'-dideoxyadenosine (ddAdo), a potent and selective inhibitor of human immunodeficiency virus (HIV) in vitro. The ddDAPR compound inhibits HIV antigen expression and HIV-induced cytopathogenicity in MT4 cells at a 50 % effective dose (ED_{50}) of 2.5-3.6 μM, as compared to 3.1-6.4 μM for ddAdo. Both compounds are endowed with a high selectivity index : 112 for ddDAPR and 139 for ddAdo. The 2',3'-unsaturated derivatives of ddDAPR and ddAdo, i.e. ddeDAPR and ddeAdo, are considerably more cytotoxic and less effective against HIV than the parental compounds. Like ddAdo, ddDAPR is only weakly inhibitory to the proliferation and DNA and RNA synthesis of a series of human B-lymphoblast, T-lymphoblast and T-lymphocyte cell lines. In contrast to ddAdo, which is rapidly deaminated by beef intestine adenosine deaminase at an initial velocity (V_1) of 145 μmol/mg protein/min, ddDAPR and ddeDAPR are poor substrates for the enzyme (V_1 : 8 and 0.7 μmol/mg protein/min, respectively), which further contributes to the potential of ddDAPR as a chemotherapeutic agent against AIDS.

Since the identification of a retrovirus, referred to as human immunodeficiency virus (HIV), as the causative agent of the acquired immunodeficiency syndrome (AIDS) (1-3), many attempts have been made to develop agents that may be useful in the prevention and/or therapy of this disease. Among the 2',3'-dideoxynucleosides, several congeners have been reported to show promising antiretroviral activity. Mitsuya et al. (4) demonstrated that 3'-azido-2',3'-dideoxythymidine (AzddThd, AZT) is a potent inhibitor of HIV replication and protects ATH8 lymphocytes against the cytopathic effect of the virus at a concentration of 5 to 10 μM. Moreover, Pauwels et al. (5) reported that AzddThd protects MT4 lymhocytes against HIV at a concentration as low as 0.02 μM, and Baba et al. (6) found that 2',3'-didehydro-2',3'-dideoxythymidine (ddeThd) and 2',3'-dideoxythymidine (ddThd) completely protect MT4 cells against HIV at 0.04 μM and 1 μM respectively. Also, Mitsuya and Broder (7) demonstrated that 2',3'-dideoxycytidine (ddCyd) was a potent inhibitor of HIV-induced cytopathogenicity in ATH8 cells, and Balzarini et al. (8) showed that 2',3'-didehydro-2',3'-dideoxycytidine (ddeCyd) had a similar antiretroviral activity as ddCyd in ATH8 cells. This observation was later confirmed by Baba et al., using MT4

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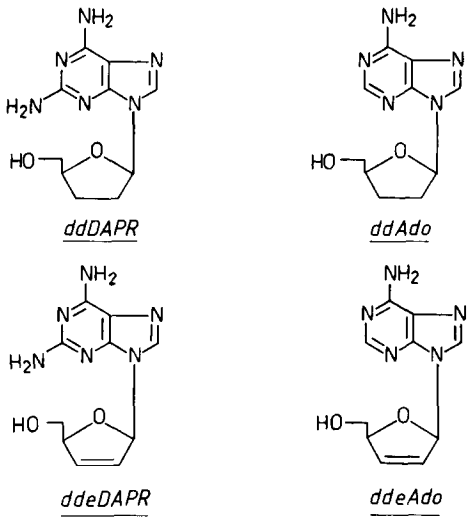

Fig. 1. Structural formulae of 2,6-diaminopurine 2',3'-dideoxyriboside (ddDAPR), 2,6-diaminopurine 2',3'-didehydro-2',3'-dideoxyriboside (ddeDAPR), 2',3'-dideoxyadenosine (ddAdo) and 2',3'-didehydro-2',3'-dideoxyadenosine (ddeAdo).

cells (6), and by Lin et al., using human peripheral mononuclear blood cells (9). Interestingly, among the purine 2',3'-dideoxyribonucleosides, 2',3'-dideoxyadenosine (ddAdo), 2',3'-dideoxyinosine (ddIno) and 2',3'-dideoxyguanosine (ddGuo) are equally effective in protecting ATH8 cells against the cytopathogenicity of HIV (7). Moreover, in ATH8 cells, the selectivity index (the ratio of the cytotoxic dose to the effective dose) was higher for ddAdo than for any of the other purine 2',3'-dideoxyriboside tested (7,10). In search of new potential inhibitors of HIV, we synthesized the 2',3'-dideoxyriboside of 2,6-diaminopurine (ddDAPR) and its 2',3'-unsaturated derivative 2,6-diaminopurine 2',3'-didehydro-2',3'-dideoxyriboside (ddeDAPR). Both ddDAPR and ddeDAPR are structurally related to ddAdo and ddeAdo (Fig. 1). We found that ddDAPR is a potent and selective inhibitor of the in vitro cytopathogenicity and infectivity of HIV, with a potency and selectivity comparable to that of ddAdo. In marked contrast with ddAdo, ddDAPR was a poor substrate for adenosine deaminase, which makes this compound even more interesting from a chemotherapeutic viewpoint.

MATERIALS AND METHODS

Compounds. 2',3'-Dideoxyadenosine was obtained from Pharmacia PL-Biochemicals. 2',3'-Didehydro-2',3'-dideoxyadenosine was synthesized according to the method of McCarthy et al. (11). The spectral data were consistent with those reported previously. 2,6-Diamino-9-(2,3-dideoxy-β-D-glycero-pent-2-enofuranosyl)purine (2,6-diaminopurine 2',3'-didehydro-2',3'-dideoxyriboside, ddeDAPR) and 2,6-diamino-9-(2,3-dideoxy-β-D-glycero-pentofuranosyl)purine (2,6-diaminopurine 2',3'-dideoxyriboside, ddDAPR) were synthesized according

to a procedure recently used for efficient conversion of adenosine into its ddeAdo derivative and hydrogenation of ddeAdo to give ddAdo (12,13). Crystalline ddeDAPR had mp 168-169°C; MS m/z 248.1020 (3.9 %, $M^+[C_{10}H_{12}N_6O_2]$ = 248.1022. Hydrogenation (11,12) of ddeDAPR gave ∿ 70 % of ddDAPR after recrystallization from methanol. Crystalline ddDAPR had mp 194-195°C; MS m/z 250.1180 (7.4 %, $M^+[C_{10}H_{14}N_6O_2]$ = 250.1178). 2,6-Diaminopurine riboside (DAPR) was synthesized by Dr. P. Herdewijn, following the method described by Gerster et al. (14) and Muraoka (15). All other reagents used were of the highest quality obtainable.

Viruses. HIV was obtained from the culture supernatant of a H9 cell line persistently infected with $HTLV\text{-}III_B$ (16).

Cells. The origin, cultivation and characterization of MT4 and Molt/4F cells used in our study have been described elsewhere (5,6,17,18). Human T4 lymphocyte H9, CEM and HUT-78 cells were cultured under the same conditions as MT4 cells.

Antiviral assays. The method for determination of the cytopathic effect of HIV in MT4 cells has previously been described (5,6). Briefly, MT4 cells were seeded at 5 x 10^5 cells/ml and infected with HIV at 50 $CCID_{50}$/ml. After 90 min incubation at 37°C, 5 x 10^5 cells were brought into wells of a flat bottomed 96-well microtiter tray containing 100 µl of various dilutions of the test compounds. After 5 days incubation at 37°C, the number of viable cells was determined microscopically in a hematocytometer by trypan blue exclusion.

Inhibition of viral antigen expression in HIV-infected MT4 cells. The inhibitory effects of the compounds on viral antigen expression in infected MT4 cells were determined at day 4 after infection by indirect immunofluorescence and laser flow cytofluorography, using a polyclonal antibody as probe, as previously described (5).

Transformation of C3H mouse embryo (MO) cells by Moloney murine sarcoma virus (Mo-MSV). MO cells were seeded into Costar Tissue Culture Cluster plates (Costar Broadway, Cambridge, Mass, USA) at 50,000 cells per ml into 2.3 cm^2 wells and grown to confluency. Cell cultures were then infected by 150 focus-forming units of Mo-MSV during 90 min, whereafter medium was replaced by 1 ml fresh culture medium containing different concentrations of test compound. After 6 days, the transformation of the cell cultures was examined microscopically.

Cytostatic and antimetabolic assays. The cytostatic effects of the compounds were assessed by measuring inhibition of cell proliferation. The inhibitory effects of the compounds on DNA and RNA synthesis of Molt/4F and H9 cells were determined by measuring inhibition of the incorporation of [methyl-^{3}H]dThd, [5-^{3}H]dCyd and [5-^{3}H]Urd into TCA-insoluble material. The experimental procedures have been described previously (18,19).

Enzyme assay. Adenosine deaminase was derived from beef intestine (Boehringer Mannheim, Mannheim, W.-Germany). The reaction mixture contained 800 µl potassium phosphate buffer 50 mM pH 7.4, 100 µl solution of the test compound (100 µM) and 100 µl (0.01 unit) of enzyme. The deamination rate was determined at room temperature by measuring the decrease in absorbancy at 265 nm for adenosine (Ado), 2'-deoxyadenosine (dAdo), ddAdo and ddeAdo and at 280 nm for ddDAPR, ddeDAPR and DAPR.

RESULTS

From Fig. 2 it is clear that ddDAPR was slightly more effective than ddAdo in protecting MT4 cells against destruction by HIV. At 5 µM, ddDAPR completely protected the cells against HIV and, at 3.6 µM, it achieved 50 % protection (Table 1). When assayed in parallel, ddAdo completely protected the cells at 25 µM, but at 5 µM, it was only partially effective. The therapeutic indexes of ddDAPR and ddAdo were as high as 112 and 139, respectively. The 2',3'-unsaturated derivatives of ddDAPR and ddAdo (i.e. ddeDAPR and ddeAdo)

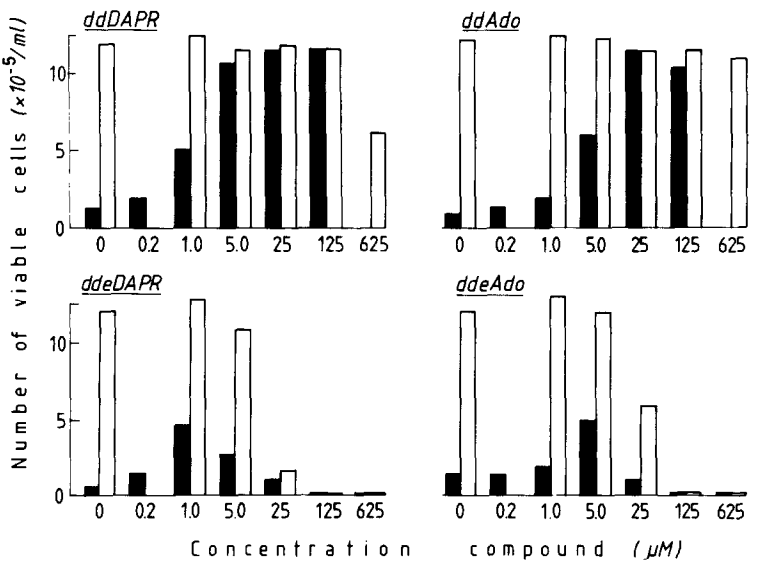

Fig. 2. Inhibition of the cytopathogenicity of HIV for MT4 cells by ddDAPR, ddeDAPR, ddAdo and ddeAdo. Viability of the cells was measured by trypan blue exclusion after an incubation period of 5 days. Mock-infected cells, incubated in the presence of different concentrations of the test compound are indicated by open columns (□), and HIV-infected cells, incubated in the presence of different concentrations of the test compounds are indicated by black columns (■). Data taken from a representative experiment.

were significantly less active and more cytotoxic than the parental compounds (Fig. 2, Table 1).

The compounds were also evaluated for their inhibitory effect on viral antigen expression in HIV-infected MT4 cells. In these experiments, ddDAPR again proved slightly superior to ddAdo, ddDAPR conferring a 50 % inhibitory effect at a concentration of 2.5 μM (Fig. 3).

TABLE 1. EFFECTIVE DOSES (ED_{50}) AND CYTOTOXIC DOSES (CD_{50}) OF ddDAPR, ddAdo, ddeDAPR AND ddeAdo

Compound	ED_{50}[a] (μM)	CD_{50}[b] (μM)	Selectivity index (CD_{50}/ED_{50})
ddDAPR	3.6	404	112
ddAdo	6.4	890	139
ddeDAPR	>1[c]	34	<34
ddeAdo	>5[c]	19	<3.4

[a] ED_{50} : dose required to achieve 50 % protection against the cell-destructive effect of HIV.
[b] CD_{50} : dose required to reduce the viability of uninfected host cells by 50 %.
[c] At these doses approximately 30-40 % protection against HIV cytopathogenicity was achieved. Because of cytotoxicity at higher doses the 50 % protective level was never attained.

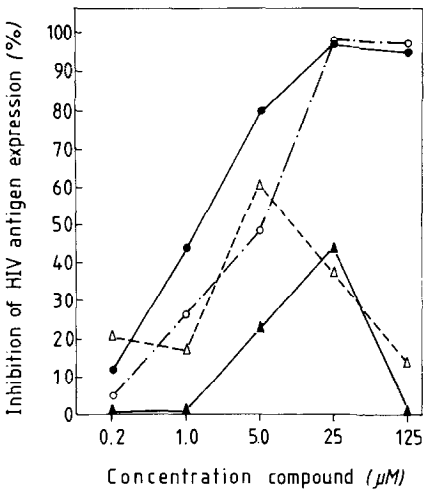

Fig. 3. Inhibition of viral antigen expression in HIV-infected MT4 cells by ddDAPR (-●-), ddAdo (-○-), ddeDAPR (-▲-) and ddeAdo (-△-). Expression of retroviral antigens was measured 4 days after infection by indirect immunofluorescence and flow cytofluorometry using polyclonal antibodies from an AIDS patient. Percent of inhibition was calculated as the ratio of number of positive cells in the presence of inhibitor to number of positive cells in the absence of inhibitor. Data taken from a representative experiment.

When examined for their inhibitory effect on the transformation of C3H mouse embryo fibroblast (MO) cells by Mo-MSV, ddDAPR and ddeDAPR were considerably more effective than ddAdo and ddeAdo. The ID_{50} (50 % inhibitory dose) values were 5 to 7 μM for ddDAPR and ddeDAPR respectively, as compared to 27 and 162 μM for ddeAdo and ddAdo, respectively (data not shown).

Next we examined the cytostatic effects of the compounds against several human (B-lymphoblast Raji, T-lymphoblast Molt/4F, and T-lymphocyte MT4, H9, HUT-78 and CEM) cell lines. As a rule ddeDAPR and ddeAdo were more cytostatic against the tumor cell lines than were their corresponding saturated counterparts (Table 2). In no case was the ID_{50} of ddDAPR and ddAdo for tumor cell proliferation lower than 100 μM. The ID_{50} of ddDAPR and ddAdo for cellular DNA

TABLE 2. INHIBITORY EFFECTS OF ddDAPR, ddAdo, ddeDAPR AND ddeAdo ON THE PROLIFERATION OF HUMAN TUMOR CELLS

Compound	ID_{50}[a] (μM)					
	Raji	Molt/4F	MT4	H9	HUT-78	CEM
ddDAPR	184	⩾ 1000	610	398	177	118
ddAdo	⩾ 1000	880	> 1000	844	650	500
ddeDAPR	90	376	48	96	110	46
ddeAdo	119	182	40	75	90	97

[a] 50 % inhibitory dose or dose required to inhibit cell proliferation by 50 %.

TABLE 3. V_i VALUES OF Ado, dAdo, ddAdo, ddDAPR, ddeAdo, ddeDAPR AND DAPR FOR BEEF INTESTINE ADENOSINE DEAMINASE

Compound	V_i[a] (μmol/min/mg protein)
Ado	192
dAdo	208
ddAdo	153
ddDAPR	8
ddeAdo	20
ddeDAPR	0.7
DAPR	60

[a]Initial velocity (V_i) was measured at an initial substrate concentration of 100 μM.

and RNA synthesis, as monitored by the incorporation of [methyl-^{3}H]dThd, [5-^{3}H]dCyd and [5-^{3}H]Urd incorporation into trichloroacetic acid-insoluble material of H9 and Molt/4F cells, was invariably higher than 500 μM (data not shown).

When examined for their susceptibilities to act as a substrate for beef intestine adenosine deaminase (ADA), the adenosine analogues showed marked differences. While ddAdo was only slightly weaker a substrate for the enzyme than were Ado and dAdo (as reflected by a slightly decreased initial velocity) (Table 3), ddDAPR was 25-fold less efficiently deaminated than Ado or dAdo. The 2',3'-unsaturated derivatives of ddAdo and ddDAPR were 8 to 13-fold less efficient substrates for ADA than their parents. The decreased rate of deamination of these compounds was also evident from their lower V_{max} values (data not shown).

DISCUSSION

The 2',3'-dideoxyriboside of 2,6-diaminopurine (ddDAPR) was slightly more effective than ddAdo in inhibiting the cytopathogenicity and antigen expression of HIV in MT4 cells. ddDAPR also had an *in vitro* therapeutic index, comparable to that of ddAdo. Both ddDAPR and ddeDAPR were superior to ddAdo and ddeAdo in inhibiting the transformation of murine fibroblasts by Mo-MSV. The fact that ddDAPR was cytostatic to human cell lines only at a concentration > 100-fold higher than the concentration required to inhibit Mo-MSV or HIV replication points to its selectivity as an antiretroviral agent. For these reasons, ddDAP must be considered as one of the most potent and selective anti-HIV agents reported so far. Furthermore, ddDAPR did not affect host cell DNA and RNA synthesis at concentrations as high as 500 μM (while inhibitory to HIV at a concentration as low as 3.6 μM).

In the light of the observations of Dalal *et al.* (20) that ddAdo is rapidly deaminated in human Molt/4F cells to 2',3'-dideoxyinosine and subse-

quently metabolized to natural adenosine nucleotides, we feel that ddDAPR, which is deaminated to a much lower extent than ddAdo by beef intestine adenosine, may be advantageous over ddAdo from a chemotherapeutic viewpoint. Moreover, when deaminated, ddDAPR is converted to ddGuo, another potent inhibitor of HIV (7). Thus, because of its relatively high resistance to deamination, ddDAPR may not only exert its antiretroviral activity as a ddDAPR metabolite (presumably ddDAPR 5'-triphosphate), but, additionally, it may also act as a prodrug of ddGuo.

I would now seem interesting to develop new 2,6-diaminopurine-2',3'-dideoxyribosides with modifications in the sugar moiety. Since we have found 3'-azido-2',3'-dideoxyadenosine (AzddAdo) to be a better substrate for adenosine deaminase than ddAdo (unpublished observations), and since AzddAdo has been reported to be an equally potent inhibitor of HIV as ddAdo (10,21), it seems imperative to synthesize 3'-azido-ddDAPR and evaluate this drug for its inhibitory effects against HIV. This drug may not only be effective as its AzddAdo metabolite but also act, if deaminated, as a prodrug of 3'-azido-ddGuo, a compound which has recently proven to be an effective inhibitor of HIV replication in vitro (22).

In conclusion, our data indicate that ddDAPR is a potent and selective inhibitor of HIV replication, and offer great potential as a chemotherapeutic agent for the treatment of retroviral infections (i.e. AIDS). Our observation that this novel drug is much less subject to deamination by adenosine deaminase than ddAdo provides an additional rationale for considering this drug as a potential antiretroviral agent in the treatment of AIDS patients.

ACKNOWLEDGEMENTS

HIV (HTLV-III_B) was kindly provided by Dr. R.C. Gallo (National Cancer Institute, Bethesda, MD, USA). MT4 cells were a gift from Dr. L. Montagnier (Pasteur Institute, Paris, France). The authors are indebted to Prof. J. Desmyter and Prof. M. Vandeputte for providing research facilities and equipment. Dr. M. Baba is a recipient of a research assistantship from the Onderzoeksfonds of the Katholieke Universiteit Leuven. Dr. P. Herdewijn is a Research Associate of the Belgian NFWO (Nationaal Fonds voor Wetenschappelijk Onderzoek). These investigations were supported in part by the AIDS Basic Research Programme of the European Community, 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), and by the Natural Sciences and Engineering Research Council of Canada, the National Cancer Institute of Canada and the University of Alberta. We thank Mrs. Danuta Madej for chemical syntheses, Ann Absillis, Miette Stuyck, Lizette Van Berckelaer and Francis Vanlangendonck for excellent technical assistance and Christiane Callebaut for fine editorial help.

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