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INVESTIGATIONS ON THE ANTI-HIV ACTIVITY OF 2′,3′-DIDEOXYADENOSINE ANALOGUES WITH MODIFICATIONS IN EITHER THE PENTOSE OR PURINE MOIETY

POTENT AND SELECTIVE ANTI-HIV ACTIVITY OF 2,6-DIAMINOPURINE 2′,3′-DIDEOXYRIBOSIDE

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Abstract—Several 2′,3′-dideoxyadenosine analogues with modifications in either the ribose or purine moiety were evaluated for their inhibitory effects on the replication of human immunodeficiency virus (HIV) in MT-4 cell cultures. The 2′,3′-dideoxyriboside of 2,6-diaminopurine (ddDAPR) inhibited HIV antigen expression and HIV-induced cytopathogenicity at a 50% effective dose of 2.4–3.8 μM, as compared to 3–6 μM for 2′,3′-dideoxyadenosine (ddAdo), whereas 50% inhibition of MT-4 cell viability was noted only at a concentration of 477 and 889 μM, respectively. Both ddDAPR and ddAdo were only weakly inhibitory to the proliferation of a number of T-lymphoblast and T-lymphocyte cell lines, pointing to the selectivity of these compounds as anti-HIV agents. In contrast to ddAdo, ddDAPR was found to be a poor substrate for adenosine deaminase, which may be advantageous from a chemotherapeutic viewpoint. Substitution of an azido or fluoro group at the 2′ and 3′- position of the ribose moiety in either "up" or "down" configurations resulted in a decrease of the anti-HIV potency and selectivity of ddAdo. In addition to ddDAPR other purine-modified ddAdo analogues, i.e. several pyrrolo[2,3-*d*]pyrimidine 2′,3′-dideoxynucleosides, were investigated for their anti-HIV activity, but none of these derivatives proved as potent or selective as ddDAPR.

Since the first cases of the Acquired Immune Deficiency Syndrome (AIDS) were diagnosed in 1981 [1–3] and the causative agent identified as a human retrovirus [4–6], now designated as human immunodeficiency virus (HIV), several compounds have been reported as active anti-HIV agents *in vitro* [7, 8]. Among these compounds are several inhibitors of the viral reverse transcriptase (RT), a key enzyme in the replicative cycle of the AIDS virus and, consequently, an attractive target for anti-HIV chemotherapy. The most potent and selective inhibitors described so far belong to the class of 2′,3′-dideoxynucleosides. They must be phosphorylated intracellularly to the 5′-mono-, -di- and -triphosphates, the latter being active as selective substrates/inhibitors of the viral RT. Lacking a free 3′-hydroxyl group these agents are assumed to act as DNA chain terminators [9]. From this group of compounds two congeners, i.e. 3′-azido-2′,3′-dideoxythymidine (AzddThd, AZT) [10] and 2′,3′-dideoxycytidine (ddCyd) [11], are currently being pursued as chemotherapeutic agents against AIDS.

In this paper, we report the anti-HIV activity of several newly-synthesized 2′,3′-dideoxyribopurine derivatives, their effect on Moloney murine sarcoma virus (MSV)-induced transformation of mouse fibroblast cells and their inhibition of the growth of a series of human and murine T-cell lines. We also describe their sensitivity to deamination by adenosine deaminase, which represents one of the major pathways by which bioactive purine nucleoside analogues are inactivated.

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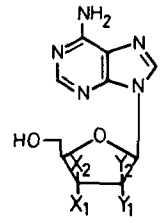
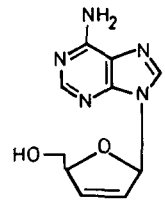
MATERIALS AND METHODS

Compounds. 2′,3′-Dideoxyadenosine (ddAdo) (Fig. 1) was obtained from Pharmacia PL-Biochemicals. 2′,3′-Didehydro-2′,3′-dideoxyadenosine (ddeAdo) (Fig. 1) was synthesized according to the method of McCarthy *et al.* [12]. The synthesis of the 2′- or 3′-fluoro- or -azido-substituted 2′,3′-dideoxyadenosine analogues (Fig. 1) will be published elsewhere [13]. 2,6-Diamino-9-(2,3-dideoxy-β-D-*glycero*-pent-2-eno-furanosyl)purine (2,6-diaminopurine 2′,3′-didehydro-2′,3′-dideoxyriboside, ddeDAPR) (Fig. 2), 2,6-diamino-9-(2,3-dideoxy-β-D-*glycero*-pentofuranosyl)purine (2,6-diaminopurine 2′,3′-dideoxyriboside, ddDAPR) (Fig. 2) and all 2′,3′-dideoxyribopyrrolo[2,3-*d*]pyrimidine derivatives (Fig. 2) were synthesized according to a procedure recently developed for efficient conversion of adenosine to ddeAdo and hydrogenation of dde-

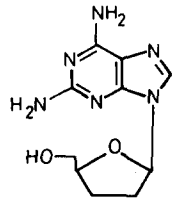


	X_1	X_2	Y_1	Y_2
dd Ado	H	H	H	H
3' - Azdd Ado	N_3	H	H	H
3' - Azdd Xylo A	H	N_3	H	H
2' - Azdd Ado	H	H	N_3	H
2' - Azdd ara A	H	H	H	N_3
3' - Fdd Ado	F	H	H	H
3' - Fdd Xylo A	H	F	H	H
2' - Fdd Ado	H	H	F	H
2' - Fdd ara A	H	H	H	F

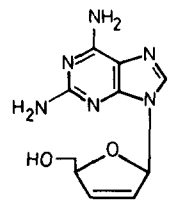


ddeAdo

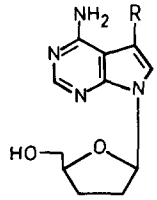
Fig. 1. Structural formulae of 2',3'-dideoxyadenosine (ddAdo), 2',3'-didehydro-2',3'-dideoxyadenosine (ddeAdo) and 2'- or 3'-substituted analogues of ddAdo.

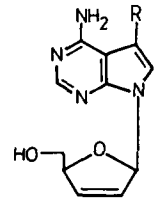


ddDAPR



ddeDAPR





	R	
dd Tub	H	dde Tub
dd Toy	CN	dde Toy
dd San	$CONH_2$	dde San

Fig. 2. Formulae of 2,6-diaminopurine 2',3'-dideoxyriboside (ddDAPR), 2',3'-dideoxytoyocamycin (ddToy), 2',3'-dideoxysangivamycin (ddSan), 2',3'-dideoxytubercidin (ddTub) and their 2',3'-unsaturated counterparts.

Ado to give ddAdo [14, 15] and will be described in more detail elsewhere. All other reagents were of the highest quality available.

Viruses. Human immunodeficiency virus (HIV) was obtained from the supernatant of a persistently HIV-infected H9 cell line (H9/HTLV-III_B) which was kindly provided by Dr R. C. Gallo, Bethesda, MD [6]. The supernatant was clarified by low speed centrifugation and stored in aliquots of known titer at −70° until used. Moloney murine sarcoma virus (MSV) was prepared from tumors induced by *in vivo* infection of 3-day-old NMRI mice as previously described [16].

Cells. The origin, cultivation and characterisation of the human T4-lymphocyte cell lines MT-4, H9, CEM and HUT 78, the human T-lymphoblast cell line Molt/4F, the human B-lymphoblast cell line Raji, the mouse leukemia cells L1210/0, the mouse mammary carcinoma cell line FM3A/0, and the murine C3H embryo fibroblasts (C3H-EF) used in this study have been described elsewhere [6, 17–19].

Anti-HIV assay in MT-4 cells. The procedure to determine the anti-HIV activity in MT-4 cells has been described previously [19]. Briefly, MT-4 cells were seeded at 5×10^5 cells per ml and infected with a freshly prepared virus dilution corresponding to 10 cc ID_{50}/microtiter well. After 90 min incubation at 37°, 5×10^4 cells were brought into the wells of a flat-bottomed 96-well microtiter tray containing 100 µl of various dilutions of the test compound. Several parameters of the infectious process were evaluated. The inhibitory effects of the compound on viral antigen expression in infected MT-4 cells were determined qualitatively at day 4 after HIV infection by indirect immunofluorescence microscopy and then quantitated by laser flow cytofluorometry, using a high titer polyclonal antibody derived from an ARC patient as probe. After 5 days incubation at 37°, the reclustering pattern of the cells, which is characteristic of normal growing MT-4 cells and completely absent in HIV-infected cells, was examined as previously described [19]. The number of viable cells in both HIV- and mock-infected cells was determined simultaneously in a blood cell counting chamber after Trypan blue staining. The 50% effective dose (ED_{50}) was defined as the concentration of compound that protected HIV-infected cells by 50%, whereas the 50% cytotoxic dose (CD_{50}) corresponded to the concentration of compound that reduced the viability of mock-infected cells by 50%.

In vitro *transformation of murine C3H embryo fibroblasts (C3H-EF) by Moloney murine sarcoma virus (MSV).* C3H-EF cells were seeded into 2.3 cm^2 wells of Costar Tissue Culture Cluster plates (Costar Broadway, Cambridge, MA, U.S.A.) at 5×10^4 cells per ml and were grown to confluency. Cell cultures were then infected with 150 focus-forming units of MSV for 90 min, whereafter the medium was replaced by 1 ml of fresh culture medium containing various concentrations of the test compounds. After 6 days, the transformation of the cell cultures was examined microscopically.

Cytostatic assays. Cytostatic effects of the compounds were assessed by measuring inhibition of cell proliferation as described previously [8, 18, 20]. Briefly, L1210/0, FM3A/0, Raji/0, Molt/4F, MT-4,

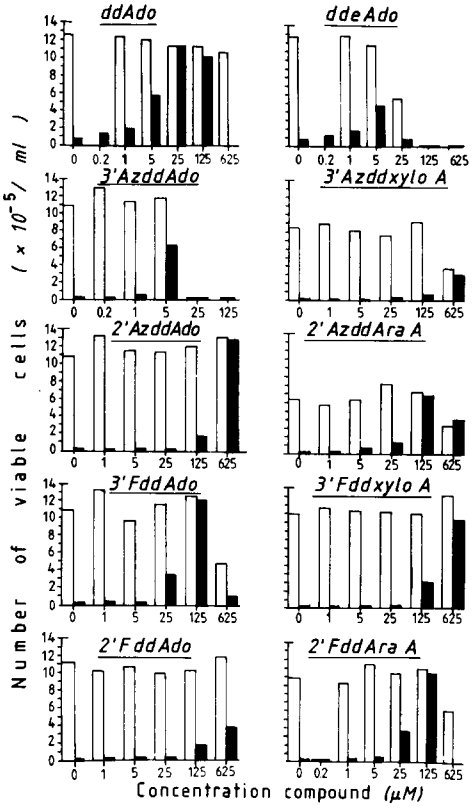


Fig. 3. Inhibition of the cytopathogenicity of HIV in MT-4 cells by 2',3'-dideoxyadenosine analogues modified in the sugar moiety. Viability of the cells was measured by the Trypan blue exclusion method on the 5th day after infection. The infected cells are indicated by solid columns (■) and mock-infected cells by open columns (□).

H9, CEM and HUT 78 cells were suspended in growth medium and added to microplate wells at a density of 5–7.5 × 10^4 cells per well in the presence of varying concentrations of the test compounds. The cells were then allowed to proliferate for 48–72 hr at 37°. At the end of the incubation period, the cells were counted in a coulter counter. The ID_{50} value was defined as the concentration of compound that reduced the cell increment by 50%.

Enzyme assay. Adenosine deaminase was derived from beef intestine (Boehringer, Mannheim, F.R.G.). 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 absorbance at 265 nm for the adenosine and pyrrolo[2,3-*d*]pyrimidine derivatives and at 280 nm for the 2,6-diaminopurine derivatives. The results are expressed as relative initial velocities of deamination with respect to adenosine.

Stability assay under acidic conditions. The compounds were incubated in 0.1 N HCl for 15 min at room temperature. After incubation, the mixture was neutralized and spotted onto TLC aluminium sheets coated with silicagel F_{254}. Separation of the nucleoside and base was achieved in chloroform:methanol (70:30).

RESULTS

A number of adenosine analogues were synthesized with either a fluorine or an azido group substituted in the "up" or "down" position at C-2' or C-3' of the sugar moiety (Fig. 1). The compounds were compared for their inhibitory effects on HIV-induced cytopathogenicity in MT-4 cells. As reference compounds were included 2',3'-dideoxyadenosine (ddAdo) and its 2',3'-unsaturated counterpart, 2',3'-didehydro-2',3'-dideoxyadenosine (ddeAdo) (Fig. 1), the former having been previously described as a potent inhibitor of HIV replication *in vitro* [11, 21]. The adenosine analogues exhibited dramatic differences in their inhibitory effects on HIV replication (Table 1), as assessed by both inhibition of HIV-induced CPE (Fig. 3) and inhibition of viral antigen expression (Fig. 5). When their CPE-inhibitory effect was recorded at day 5 post-infection, the compound with the 3'-azido "down" was found to be the most potent with an ED_{50} of 5 μM which is comparable to ddAdo (ED_{50}:6.2 μM). 3'-AzddAdo reduced antigen expression in HIV-infected MT-4 cells by 50% at a concentration of 13 μM, although it did not completely suppress viral protein expression at a concentration of 125 μM (Fig. 5). In addition, this

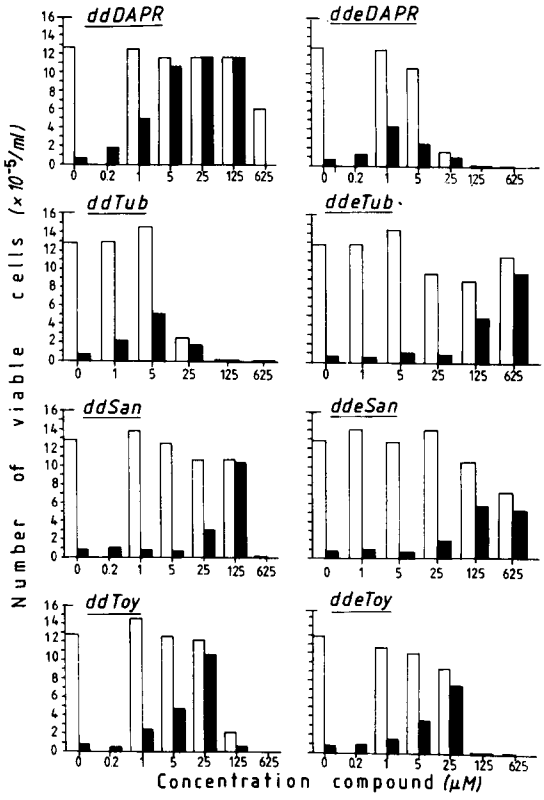


Fig. 4. Inhibition of the cytopathogenicity of HIV in MT-4 cells by 2',3'-dideoxyadenosine and 2',3'-didehydro-2',3'-dideoxyadenosine analogues modified in the base moiety. Viability of the cells was measured by the Trypan blue exclusion method on the 5th day after infection. The infected cells are indicated by solid columns (■) and the mock-infected cells by open columns (□).

Table 1. Anti-HIV activity and cytotoxicity of 2',3'-dideoxyadenosine analogues, 2,6-diaminopurine 2',3'-dideoxyriboside and its unsaturated counterpart, and the 2',3'-dideoxy derivatives of tubercidin, toyocamycin and sangivamycin

Compound	CD_{50} (μM)*	ED_{50} (μM)†		Selectivity index‡
		CPE§	Immunofluorescence‖	
ddAdo	889	6.2	3	143
ddeAdo	34	>25	3.6	<1.2
3'-AzddAdo	10	5	13	2
3'-AzddxyloA	551	>625	>125	<0.9
2'-AzddAdo	>625	215	>125	>2.9
2'-AzddaraA	625	55	25	11.4
3'-FddAdo	557	50	78	11.1
3'-FddxyloA	>1250	271	43	>4.6
2'-FddAdo	>625	>625	>125	><1
2'-FddaraA	>625	35	20	>18
ddDAPR	477	3.8	2.4	125
ddeDAPR	35	>125	>25	<1
ddToy	54	8.5	2.3	6.3
ddeToy	44	14	10	3.1
ddSan	242	64	56	3.8
ddeSan	625	>125	78	<5
ddTub	33	>25	—	<1.3
ddeTub	>1250	205	30	>6.1

* Dose required to reduce the viability of uninfected host cells by 50% after 5 days incubation in the presence of the compound.

† 50% antiviral effective dose.

‡ Ratio of CD_{50} to ED_{50} (based upon CPE assay).

§ CPE assay based on cell viability determined 5 days after HIV infection.

‖ Immunofluorescence assay based on HIV antigen expression determined by quantitative laser flow cytofluorometry 4 days after HIV infection.

All data represent average values for at least two separate experiments.

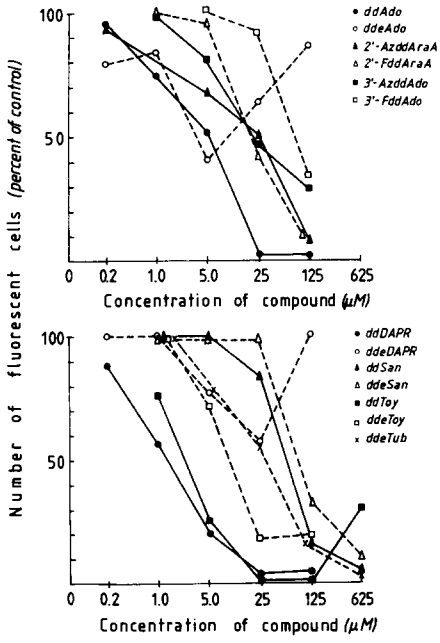


Fig. 5. Inhibition of viral antigen expression in HIV-infected MT-4 cells by various 2',3'-dideoxyadenosine analogues modified in the base and/or sugar moiety. The number of antigen-positive cells, based on the accumulated data of 10^4 cells, was measured by indirect immunofluorescence and laser flow cytofluorometry, using a polyclonal antibody as probe.

compound proved about 100-fold more cytotoxic for the host cells than the parent compound (CD_{50}:10 μM vs 889 μM). The adenosine analogue with an azido substituent in the C-2' "up" position was only slightly toxic (CD_{50}:625 μM); however, this compound was about 10-fold less active against HIV than ddAdo, as determined by both the CPE and antigen expression assays. Neither the C-2' azido "down" nor the C-3' azido "up" resulted in an appreciable anti-HIV activity. Similar observations were made for the fluorine-substituted series. The highest toxicity was seen for the compound with the fluorine atom in the C-3' "down" position, whereas the greatest anti-HIV selectivity was observed with the C-2' fluoro "up" analogue. However, none of the 2'- or 3'-fluoro- or -azido-substituted derivatives of ddAdo even approached the parent compound in anti-HIV selectivity.

We then evaluated several ddAdo derivatives in which an additional amino group was introduced in the 2-position of the purine ring (Fig. 2) or N-7 was substituted by either CH (tubercidin), CCN (toyocamycin), or $CCONH_2$ (sangivamycin) (Fig. 2). The anti-HIV properties of these compounds are presented in Table 1. The most active and most selective anti-HIV agent of this class of compounds was ddDAPR as illustrated in Fig. 4. At 5 μM, ddDAPR completely protected MT-4 cells against the CPE of HIV, which makes this compound slightly more potent than ddAdo. At this concentration, "reclustering" of the MT-4 cells, a property which is

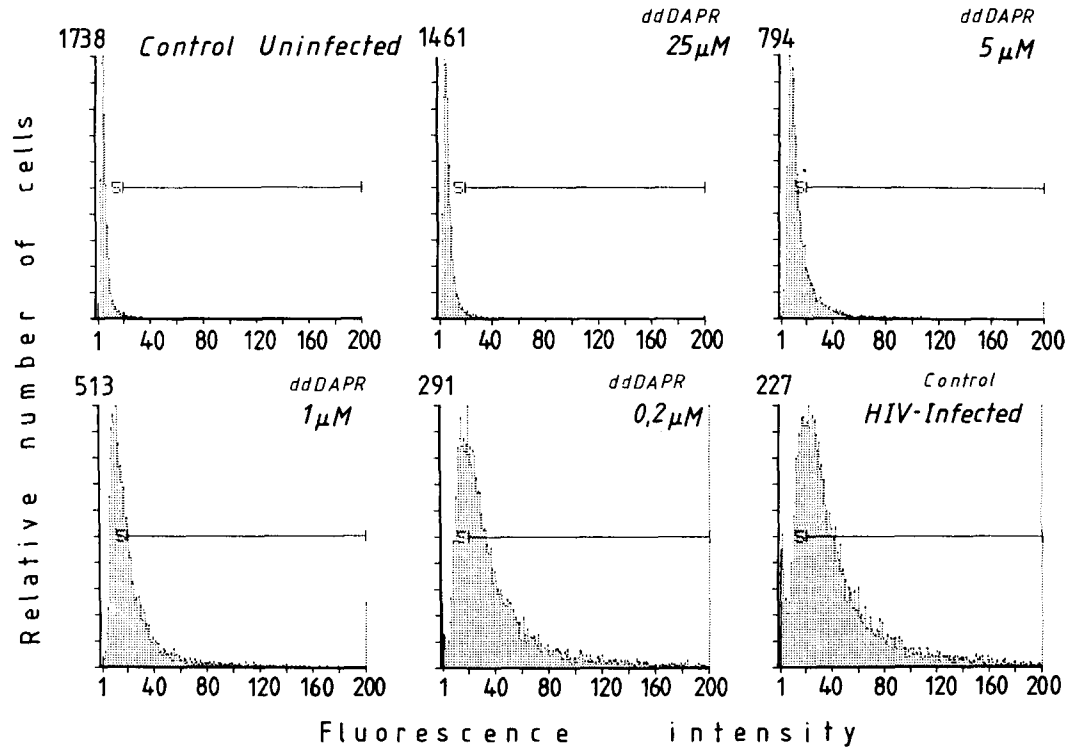


Fig. 6. Laser flow cytofluorometric histogram analysis of HIV-infected MT-4 cells treated with varying concentrations of ddDAPR and then processed by the indirect immunofluorescence method using polyclonal antibody as probe. The values indicated on the top of the *y*-axis represent the total numbers of cells scored.

completely lost for untreated HIV-infected cells, could again be observed (data not shown). In addition, viral antigen expression was completely inhibited at a ddDAPR concentration of 25 μM and signficantly diminished within the concentration range of 1–5 μM, as assayed by both indirect immunofluorescence microscopy (data not shown) and laser flow cytofluorometric histogram analysis (Figs 5 and 6). The inhibitory effects of ddDAPR were not reversed when the cells were incubated in the presence of the natural purine (i.e. Ado, dAdo) or pyrimidine (i.e. dThd, dCyd) nucleosides (data not shown). The 2′,3′-didehydro derivative of ddDAPR (ddeDAPR) was significantly less active and more cytotoxic than the parent compound as was the 2′,3′-didehydro derivative of ddAdo (ddeAdo) *vis-à-vis* ddAdo. From the pyrrolo[2,3-*d*]pyrimidine series (Table 1, Fig. 4), the toyocamycin derivative ddToy exerted the most marked anti-HIV activity with an ED_{50} for the inhibition of CPE and viral antigen expression of 8.5 and 2.3 μM, respectively. The compound effected a 50% reduction in MT-4 cell viability at a concentration of 54 μM (Table 1). Except for ddeTub, which showed little, if any, toxicity and yet some anti-HIV activity, none of the other pyrrolo[2,3-*d*]pyrimidine derivatives examined proved to be significantly selective HIV inhibitors.

Effects of purine 2′,3′-dideoxynucleoside analogues on in vitro *transformation of C3H-EF cells by MSV*

In addition to their anti-HIV activity, we explored the antiviral activity of these compounds in another retroviral system based on the transformation of mouse embryo cells by MSV (Table 2). ddDAPR and its unsaturated congener, ddeDAPR, showed a potent and selective inhibition of MSV-induced transformation with ED_{50} values of 5.8 and 4 μM, respectively, and selectivity indexes greater than 10. For ddAdo to inhibit MSV focus formation an ED_{50} of 128 μM was required, whereas ddeAdo showed an ED_{50} of 27 μM in this assay system. Of the 2′- or 3′-fluoro- or -azido-substituted 2′,3′-dideoxyadenosine analogues, only the compound with the 3′-azido "down" had some anti-MSV activity (ED_{50}:69 μM). Of the pyrrolo[2,3-*d*]pyrimidine derivatives, only ddeTub showed an appreciable activity without cytotoxicity in the MSV assay system; its ED_{50} value being 27 μM. The other pyrrolo[2,3-*d*]pyrimidine derivatives were not selectively inhibitory towards MSV focus formation (Table 2).

Cytostatic effects of purine 2′,3′-dideoxynucleoside analogues on human and murine tumor cell lines

We examined the cytostatic effects of the 2′- and 3′-substituted adenosine analogues, the 2,6-diaminopurine derivatives and the pyrrolo[2,3-*d*]pyrimidine nucleosides against several murine (leukemia L1210/0, mammary carcinoma FM3A) and human (B-lymphoblast Raji, T-lymphoblast Molt/4F and T-lymphocyte MT-4, H9, CEM and HUT-78) cell lines. For ddAdo the 50% inhibitory dose for cell proliferation ranged from about 500 μM in CEM cells to over 1000 μM in MT-4 cells, whereas a wider range of ID_{50} values was noted for ddDAPR (ID_{50}:119 μM

Table 2. Effects of various purine 2′,3′-dideoxynucleoside analogues on Moloney murine sarcoma virus-induced transformation of C3H-EF cells

Compound	Minimum cytotoxic concentration* (μM)	ED_{50} (μM)†
ddAdo	>200	128
ddeAdo	>200	27
3′-AzddAdo	>200	69
3′-AzddxyloA	>200	>200
2′-AzddAdo	>200	>200
2′-AzddaraA	>200	>200
3′-FddAdo	>200	>200
3′-FddxyloA	>200	>200
2′-FddAdo	>200	>200
2′-FddaraA	>200	>200
ddDAPR	>200	5.8
ddeDAPR	≥40	4
ddToy	≥1.6	>1.6
ddeToy	≥8	>1.6
ddSan	≥40	>40
ddeSan	200	>40
ddTub	≥8	4.8
ddeTub	>200	27

* Dose required to cause a microscopically detectable alteration of normal cell morphology.

† Dose required to reduce the number of MSV-induced foci by 50%.

All data represent average values for at least two separate experiments.

for CEM cells, as compared to 1000 μM for Molt/4F cells). The 2′,3′-didehydro derivatives ddeAdo and ddeDAPR were significantly more cytostatic against human cell lines than were their saturated counterparts (Table 3). However, within the pyrrolo[2,3-*d*]pyrimidine nucleoside series, a reverse relationship was found, with 2′,3′-dideoxytubercidin being on the average at least 10-fold more cytostatic than its 2′,3′-dideoxy-2′,3′-didehydro counterpart. No differences were noted between murine and human tumor cells in their susceptibility to the cytostatic effects of ddTub and its congeners. 3′-AzddAdo, however, exerted somewhat lower cytostatic effects on murine than on human tumor cell lines (Table 3).

Susceptibility to deamination by adenosine deaminase and to N*-glycosidic cleavage under acidic conditions*

To assess their susceptibility to deamination, we evaluated the initial velocities of deamination by beef intestine adenosine deaminase (ADA) for all compounds tested (Table 4). In comparison with Ado and ddAdo, the initial velocity of deamination for ddDAPR was about 25-fold and for ddeDAPR even 300-fold lower, whereas for ddeAdo the initial rate of deamination was decreased by about 10-fold. Within the 2′- or 3′-fluoro- or -azido-substituted adenosine series, the compounds with the 3′-fluoro substituents showed the weakest susceptibility to deamination. In general, all 2′- and 3′-fluoro- and -azido-substituted ddAdo analogues proved to be good substrates for adenosine deaminase (Table 4). Interestingly, 3′-AzddAdo (azido "up" or "down") and 2′-FddAdo (fluoro "down") were even better substrates for adenosine deaminase than was ddAdo. Of the pyrrolo[2,3-*d*]pyrimidine analogues, none was a substrate for ADA as expected.

The two most selective anti-HIV agents among the 2′,3′-dideoxyadenosines, namely ddAdo and 2′-FddaraA (Table 1) were also evaluated for their susceptibility to *N*-glycosidic cleavage under acidic conditions (pH 1.0). At this pH, ddAdo was completely degraded to adenine whereas 2′-FddaraA did not show any degradation at all (data not shown).

DISCUSSION

In addition to the efforts in developing a vaccine against HIV which might ultimately protect those not yet affected by the AIDS virus, a world wide search for an effective antiviral chemotherapy has been launched. This approach has been prompted by the assumption that continued HIV replication is involved in both the pathogenesis and the progression of the disease [22, 23]. Further support for this approach stems from the initial placebo-controlled, randomized clinical trial carried out with the 3′-azido derivative of 2′,3′-dideoxythymidine (3′-AzddThd, AZT), which has shown that treatment with this drug may result in an improvement of the clinical, virological and immunological parameters of the disease [24, 25].

By the various research groups now involved in anti-HIV drug development, a number of different target cell lines and methodologies are being used, which makes a comparison of the data less than straightforward. In particular the multiplicity of virus infection, the sensitivity of the cell lines used, the duration of the assay and the cell-dependent differences in drug metabolism may influence the data obtained. Metabolic conversions are of particular importance for the 2′,3′-dideoxynucleosides as their 5′-triphosphate forms are targeted at the reverse transcriptase (RT) of HIV and therefore need to be phosphorylated by several host cellular kinases. The toxicity of these compounds is assumed to result from the possible interaction of the 5′-triphosphates with cellular DNA polymerases. However, interaction of the nucleosides or their phosphorylated products with one or more other steps involved in nucleoside and/or nucleotide metabolism may well contribute to their cytotoxicity.

Our anti-HIV assay system is based on a T4 cell line (MT-4) which has exquisite sensitivity towards HIV infection. The MT-4 cells are infected with a viral dose that completely destroys all target cells after 5 days incubation and yet allows the virus to go through several replicative cycles It should also be pointed out that the test compounds are added immediately after virus adsorption. Under these conditions we have previously shown that 2′,3′-dideoxycytidine (ddCyd) completely protects MT-4 cells against the cytopathic effect of HIV at a concentration of 0.5 μM [19, 26], 2′,3′-didehydro-2′,3′-dideoxycytidine (ddeCyd) at 1 μM [27], ddAdo and 2′,3′-dideoxyguanosine (ddGuo) each at 25 μM [28] and 3′-azido-2′,3′-dideoxyguanosine (AzddGuo) at 5 μM [29]. These values correspond well with those reported in the literature [11, 29–31]

However, of all nucleoside analogues that have been evaluated so far in our laboratory for their

Table 3. Cytostatic effects of various purine 2',3'-dideoxynucleoside analogues on a series of murine and human cell lines

Compound	ED_{50} (μM)*							
	L1210/0†	FM3A0‡	Raji§	MOLT/4F‖	MT-4¶	H9¶	CEM¶	HUT78¶
ddAdo	—	—	953	887	>1000	875	508	654
ddeAdo	148	264	118	183	40	75	96	90
3'AzddAdo	74	97	6	18	17	25	13	20
3'-AzddxyloA	>200	>200	>200	>200	>200	>200	—	—
2'-AzddAdo	>200	>200	>200	>200	>200	>200	—	—
2'-AzddaraA	>200	>200	>200	>200	>200	>200	—	—
3'-FddAdo	339	996	382	810	>200	>200	—	—
3'-FddxyloA	>200	>200	>200	>200	178	>200	—	—
2'-FddAdo	≥3970	≥3970	≥3970	≥3970	>1000	>1000	—	—
2'-FddaraA	>200	>200	>200	>200	>200	>200	—	—
ddDAPR	—	—	183	>1000	610	398	119	177
ddeDAPR	—	—	91	376	48	96	46	110
ddToy	33	>38	29	>38	16	>38	—	—
ddeToy	>39	>39	>39	>39	>39	>39	—	—
ddSan	165	>181	141	>181	>181	>181	—	—
ddeSan	>364	>364	>364	>364	>364	>364	—	—
ddTub	11	21	16	22	12	13	—	—
ddeTub	>216	>216	>216	>216	>216	>216	—	—

* Dose required to inhibit cell proliferation by 50%; data represent the average of at least three separate experiments.
† Murine leukemia cells.
‡ Murine mammary carcinoma cells.
§ Human B-lymphoblasts.
‖ Human T-lymphoblasts.
¶ Human T4-lymphocytes.

ability to inhibit the *in vitro* replication and cytopathic effect of HIV in MT-4 cells, the pyrimidine analogues 3'-azido-2',3'-dideoxythymidine (3'-AzddThd, AZT) (the anti-HIV activity of which was first mentioned by Mitsuya *et al.* ([10]) and the 2',3'-unsaturated derivative of thymidine (ddeThd) emerged as the most potent on a molar basis. They inhibited HIV replication at concentrations of about 0.04 μM [19, 30]. The increased anti-HIV potency and selectivity noted with some thymidine analogues in MT-4 cells [29, 32] as compared with, for instance, ATH8 cells [10, 11], may be related to an increased conversion rate of the thymidine nucleosides to the 5'-triphosphates. That there exist remarkable differences in the phosphorylation rate of thymidine analogues from one cell species to another has been recently shown by Balzarini *et al.* [33] for AZT. However, the latter study also showed that increased levels of AZT 5'-triphosphate did not correlate with the cytostatic activity of the compound.

Table 4. Relative initial velocities of deamination by beef intestine adenosine deaminase with respect to adenosine

Substrate	Relative velocity
Ado	1.00
dAdo	1.08
ddAdo	0.79
ddeAdo	0.10
3'-AzddAdo	1.59
3'-AzddxyloA	ND
2'-AzddAdo	1.32
2'-AzddaraA	ND
3'-FddAdo	0.47
3'-FddxyloA	0.41
2'-FddAdo	1.34
2'-FddaraA	ND
ddDAPR	0.042
ddeDAPR	0.003
ddToy	0
ddeToy	0
ddSan	0
ddeSan	0
ddTub	0
ddeTub	0

ND: not determined.

The 2',3'-dideoxyriboside of 2,6-diaminopurine (ddDAPR) proved to be as potent and selective in inhibiting HIV as did ddAdo, one of the most selective anti-HIV agents reported so far [11, 34]. The antiretroviral activity of ddDAPR extended to murine systems where the compound was found to inhibit MSV-induced transformation of mouse embryo fibroblast cells at 5.8 μM, that is at a 20-fold lower dose than ddAdo. Cooney *et al.* [34] have recently shown that ddAdo is inefficiently anabolized to its 5'-triphosphate and its potential to inhibit HIV replication is largely impaired by its extensive deamination to 2',3'-dideoxyinosine (ddIno) which is then cleaved by purine nucleoside phosphorylase to release hypoxanthine.

In contrast with ddAdo, ddDAPR appears to be a very poor substrate for adenosine deaminase (Table 4) [35, 36]. However, if the compound were to be deaminated, 2',3'-dideoxyguanosine would be generated, which, as shown by Mitsuya *et al.* [11] and Baba *et al.* [28], is still inhibitory to HIV, albeit

with a somewhat smaller therapeutic ratio than ddAdo. Thus, ddDAPR merits further evaluation for its efficacy in the treatment of retrovirus infections. Furthermore, the 3′-azido derivative of ddDAPR should also be pursued for its anti-HIV properties. Hartmann *et al.* [37] and Baba *et al.* [28] have shown that the 3′-azido derivative of ddGuo is equally active, if not more so, in inhibiting HIV replication than ddGuo is, and it would seem interesting to verify whether this observation extends to ddDAPR which could be considered as a putative prodrug of ddGuo.

When 2′,3′-unsaturation was introduced in the sugar part of ddDAPR, the compound became much more toxic for various cell lines whereas its anti-HIV activity was annihilated; although in the murine system its anti-MSV activity remained essentially unaltered. The 2′,3′-didehydro derivatives of ddAdo and ddGuo followed the same trend, in that, like ddeDAPR, ddeAdo and ddeGuo were virtually devoid of any selective anti-HIV activity [28, 29]. The pyrrolo[2,3-*d*]pyrimidine derivatives did not seem to follow this rule. In addition, the therapeutic margin of these compounds was rather narrow.

While none of the 2′- or 3′-fluoro- or -azido-substituted 2′,3′-dideoxyadenosine analogues proved more potent or selective against HIV than ddAdo, a structure–function relationship analysis revealed the highest selectivity for those compounds that contained the fluorine or azido substituent in the 2′-ara ("up") configuration. This may be of potential value in the design of novel derivatives of 2′,3′-dideoxyadenosine. Moreover, the resistance of the C-2′ fluoro "up" analogue to degradation under acidic conditions may be relevant in terms of bioavailability after oral administration.

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