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THE 2',3'-DIDEOXYRIBOSIDE OF 2,6-DIAMINOPURINE AND ITS 2',3'-DIDEHYDRO DERIVATIVE INHIBIT THE DEAMINATION OF 2',3'-DIDEOXYADENOSINE, AN INHIBITOR OF HUMAN IMMUNODEFICIENCY VIRUS (HIV) REPLICATION

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The 2',3'-dideoxyriboside of 2,6-diaminopurine (ddDAPR) and its 2',3'-didehydro derivative (ddeDAPR) are poor substrates for adenosine deaminase (ADA) but potent inhibitors of the enzyme. Their K_m values for ADA are of the same order of magnitude as those of the natural adenosine (Ado) and 2'-deoxyadenosine (dAdo), but their V_{max} values are 35-fold (ddDAPR) to 350-fold (ddeDAPR) lower than those of Ado and dAdo. The K_i/K_m values of ADA for ddeDAPR (as inhibitor) and Ado, 2',3'-dideoxyadenosine (ddAdo) and 9-β-D-arabinofuranosyladenine (araA) as the substrates are 0.17, 0.05 and 0.06, respectively. ddDAPR is about 3-fold less potent as an inhibitor of ADA than ddeDAPR. The 2,6-diaminopurine derivatives ddeDAPR and ddDAPR [which is also a potent inhibitor of human immunodeficiency virus (HIV)], may hold great promise, from a chemotherapeutic viewpoint, in combination with other adenosine analogues such as ddAdo and araA, which have been recognized and/or being pursued as either anti-retrovirus or anti-herpesvirus agents.

Acquired immunodeficiency syndrome (AIDS) is caused by a retrovirus designated as human immunodeficiency virus (HIV) (1-4). Recently, it has been shown that 2',3'-dideoxyribosides of several purines (i.e. adenine, guanine, hypoxanthine) are effective in protecting ATH8 cells against the infectivity and cytopathogenicity of HIV *in vitro* (5). In ATH8 cells, the selectivity index (the ratio of the cytotoxic dose to the effective dose) of 2',3'-dideoxyadenosine (ddAdo) was higher than that of any of the other 2',3'-dideoxyriboside tested (6,7). Adenosine (Ado) analogues have since long generated interest in cancer chemotherapy, immunology and virology (8). One of the factors which limits the therapeutic usefulness of Ado analogues such as 9-β-D-arabinosyladenine (araA) is their rapid degradation by adenosine deaminase (ADA) (8,9). From the study of Bloch *et al.* (10) on the susceptibility of Ado analogues to ADA it appeared that ddAdo is a good substrate for ADA (K_m = 77 μM), the initial velocity (V_i) for the enzymatic reaction being ∿ 60 % of the V_i for the natural substrate Ado.

Recently, we have found that 2,6-diaminopurine 2',3'-dideoxyriboside (ddDAPR) is a potent and selective inhibitor of HIV-induced cytopathogenicity

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in MT4 cells (11). This novel compound is a structural analogue of ddAdo in which an amino group is substituted at C-2 of the purine ring (Fig. 1). We found that, in contrast to ddAdo which is rapidly deaminated by beef intestine adenosine deaminase at an initial velocity of 145 μmol/mg protein/min, ddDAPR and its 2',3'-didehydro derivative ddeDAPR are poor substrates for the enzyme (V_i : 8 and 0.7 μmol/mg protein/min, respectively). These characteristics may contribute to the potential of ddDAPR as a chemotherapeutic agent against AIDS. In this report we describe the kinetic properties of ADA towards ddDAPR and ddeDAPR as well as Ado, ddAdo, araA, 2'-deoxyadenosine (dAdo), 2',3'-didehydro-ddAdo (ddeAdo), 2,6-diaminopurine riboside (DAPR) and 2,6-diaminopurine 2'-deoxyriboside (dDAPR). We found that ddDAPR and ddeDAPR were not only poor substrates for ADA (with V_{max} values that were 35- to 350-fold lower than those noted for Ado and dAdo), but also strong inhibitors of the deamination of ddAdo and araA. These findings make ddDAPR and ddeDAPR interesting candidates for combination therapy with ddAdo or araA, i.e. in the treatment of either retrovirus or herpesvirus infections, or both.

MATERIALS AND METHODS

Compounds. Adenosine (Ado), 2'-deoxyadenosine (dAdo), 9-β-D-arabinofuranosyladenine (araA) and 2,6-diaminopurine 2'-deoxyriboside (dDAPR) were obtained from Sigma Chemical Company (Milwaukee, Wisconsin, USA) and 2',3'-dideoxyadenosine (ddAdo) was purchased from Pharmacia PL-Biochemicals. 2',3'-Didehydro-2',3'-dideoxyadenosine (ddeAdo) was synthesized according to the method of Mc Carthy et al. (12). 2,6-Diaminopurine riboside (DAPR) was synthesized by Dr. P. Herdewijn, following the method described by Gerster et al. (13) and Muraoka (14). 2,6-Diamino-9-(2,3-dideoxy-β-D-glycero-pent-2-enofuranosyl)purine (ddeDAPR) and 2,6-diamino-9-(2,3-dideoxy-β-D-glycero-pentofuranosyl)purine (ddDAPR) were synthesized by Prof. M.J. Robins according to a procedure recently used for conversion of Ado into its ddeAdo derivative and hydrogenation of ddeAdo to give ddAdo (15,16). The detailed procedure will be published elsewhere.

Enzyme. Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) was prepared from beef intestine, and provided by Boehringer Mannheim (Mannheim, W.-Germany) as a suspension in ammoniumsulfate solution, 3.2 mol/liter, pH 6.0. The enzyme preparation had a specific activity of ca. 200 U/mg (25°C; adenosine as substrate), and was contaminated with alkaline phosphatase, adenylate deaminase, guanase and nucleoside phosphorylase for less than 0.01 %.

Enzymatic assay. The enzyme was diluted in bidistilled water to a concentration of 0.1 unit/ml in the assays with Ado, dAdo and ddAdo, to 0.5 units/ml in the assays with araA, ddeAdo, DAPR and dDAPR and to 10 units/ml in the assays with ddDAPR and ddeDAPR. The experiments were performed by adding 0.1 ml enzyme solution to 0.9 ml each of different concentrations of substrate in 50 mM potassium phosphate buffer pH 7.5 at room temperature. The compound solution was added to the sample cuvette; enzyme was added to both sample and reference cuvettes. The rates of deamination at the two highest substrate concentrations were almost identical, indicating that the enzyme was essentially saturated at these levels. Progress of the deamination was recorded for Ado, dAdo, ddAdo, ddeAdo and araA at 265 nm, and for DAPR, dDAPR, ddDAPR and ddeDAPR at 280 nm. The K_m and V_{max} values were determined by the Lineweaver and Burk procedure using a linear regression program. For the inhibition studies, compound solution (i.e. Ado, ddAdo, araA) was added to the sample cuvette only, and the inhibitor solution (i.e. ddDAPR, ddeDAPR) was added to

both the sample and reference cuvettes. The final enzyme concentration was 0.01 unit/ml for Ado and ddAdo, and 0.05 unit/ml for araA. At these enzyme dilutions, no significant deamination of ddDAPR and ddeDAPR occurred within the incubation time of the assay.

RESULTS

All nine adenosine analogues (Fig. 1) were examined for their susceptibility to deamination by beef intestine adenosine deaminase (ADA). The K_m values of ADA for the natural substrates Ado and dAdo were 29 μM and 15 μM, respectively (Table 1). A similar K_m value was observed for the potent anti-HIV drug ddDAPR (K_m : 29 μM), while a 2.5-fold higher K_m value was noted for ddAdo (K_m : 73 μM). The 2',3'-didehydro derivatives of ddAdo and ddDAPR, i.e. ddeAdo and ddeDAPR, showed a 2.5- to 3.5-fold higher affinity for ADA than the parental compounds. However, for some adenosine analogues, i.e. ddeAdo, ddDAPR and

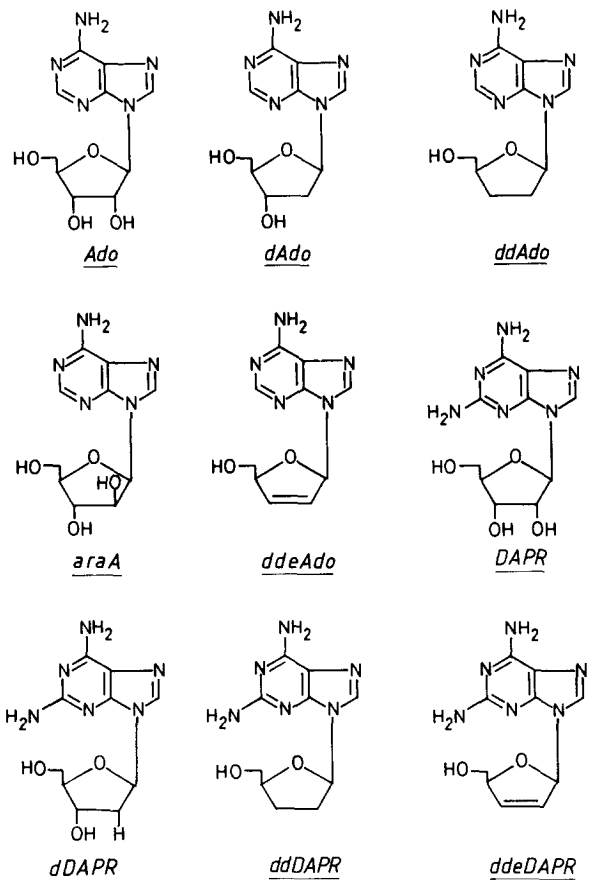

Fig. 1. Structural formulae of adenosine (Ado), 2'-deoxyadenosine (dAdo), 2',3'-dideoxyadenosine (ddAdo), 2',3'-didehydro-2',3'-dideoxyadenosine (ddeAdo), 9-β-D-arabinofuranosyladenine (araA), 2,6-diaminopurine riboside (DAPR), 2,6-diaminopurine 2'-deoxyriboside (dDAPR), 2,6-diaminopurine 2',3'-dideoxyriboside (ddDAPR), 2,6-diaminopurine 2',3'-didehydro-2',3'-dideoxyriboside (ddeDAPR).

TABLE 1. Kinetic properties of beef intestine adenosine deaminase for adenosine analogues

Compound	K_m (μM)	V_{max} (μmole/mg protein/min)	V_{max}/K_m
Ado	29	287	9.9
dAdo	15	232	15
ddAdo	73	262	3.6
ddeAdo	30	27.2	0.9
araA	73	75	1.1
DAPR	22	89	4.0
dDAPR	16	88	5.5
ddDAPR	29	8.23	0.28
ddeDAPR	8	0.754	0.09

ddeDAPR, the V_{max} values were by several orders of magnitude lower than those for Ado, dAdo and ddAdo (Table 1). The V_{max} of Ado, dAdo and ddAdo was 287, 232 and 262 μmol/mg protein/min, respectively, while with ddeDAPR deamination at saturating substrate concentrations did not exceed a rate of 0.754 μmol/mg protein/min, which is 350-fold lower than the rate noted for Ado, dAdo or ddAdo. As a rule, the V_{max} values of the 2',3'-didehydro derivatives of ddAdo and ddDAPR, i.e. ddeAdo and ddeDAPR, were 10-fold lower than those of their 2',3'-dihydrogenated counterparts. This observation corresponds well with that of Bloch *et al.* (10), who reported that the deamination of ddeAdo by ADA from intestinal mucosa proceeded at a 6-fold lower rate than the deamination of ddAdo. Assuming that the V_{max}/K_m ratio can be considered as a parameter for the efficiency of deamination of adenosine analogues, Ado would be deaminated 100 times faster than ddeDAPR (Table 1). It is also of particular interest to note that ddAdo is converted to 2',3'-dideoxyinosine (ddIno) at a 10-fold faster rate than ddDAPR to 2',3'-dideoxyguanosine (ddGuo), all four 2',3'-dideoxynucleosides having been recognized as potent anti-HIV agents (5,11).

Based on the observation that the V_{max} for ddDAPR and ddeDAPR was 35- to 350-fold lower than the V_{max} for ddAdo, we examined the effect of ddDAPR and ddeDAPR on the deamination of Ado, ddAdo and araA. The K_i and K_i/K_m values of ADA for ddDAPR and ddeDAPR with Ado, ddAdo and araA as the substrate are presented in Table 2 and double-reciprocal plots are depicted in Fig. 2. The most potent inhibitor of ADA was ddeDAPR. The K_i/K_m value for ddeDAPR varied from 0.05 to 0.17. For ddDAPR it varied from 0.19 to 0.48. For both inhibitors, the type of inhibition appeared to be competitive with respect to Ado, ddAdo and araA (Fig. 2). The strongest inhibitory effect was noted with ddeDAPR on the deamination of ddAdo and araA. No significant differences in the kinetics of the inhibitors were noted upon preincubation of ddDAPR and ddeDAPR with the enzyme for 30 minutes prior to the addition of ddAdo as substrate (data not shown).

TABLE 2. K_i values of beef intestine adenosine deaminase for ddDAPR and ddeDAPR, with Ado, ddAdo and araA as the substrate

Substrate	Inhibitor			
	ddDAPR		ddeDAPR	
	K_i	K_i/K_m	K_i	K_i/K_m
Ado	14.1	0.48	5.2	0.17
ddAdo	14.1	0.19	3.8	0.05
araA	20.6	0.23	5.4	0.06

DISCUSSION

Only a few very potent adenosine deaminase inhibitors have so far been reported. They include coformycin (CF), isocoformycin (ICF), 2'-deoxycoformycin (DCF), erythro-9-(2-hydroxyl-3-nonyl)adenine (EHNA) and several derivatives of EHNA. Their K_i values for ADA are within the 10^{-9} to 10^{-12} M range (9,

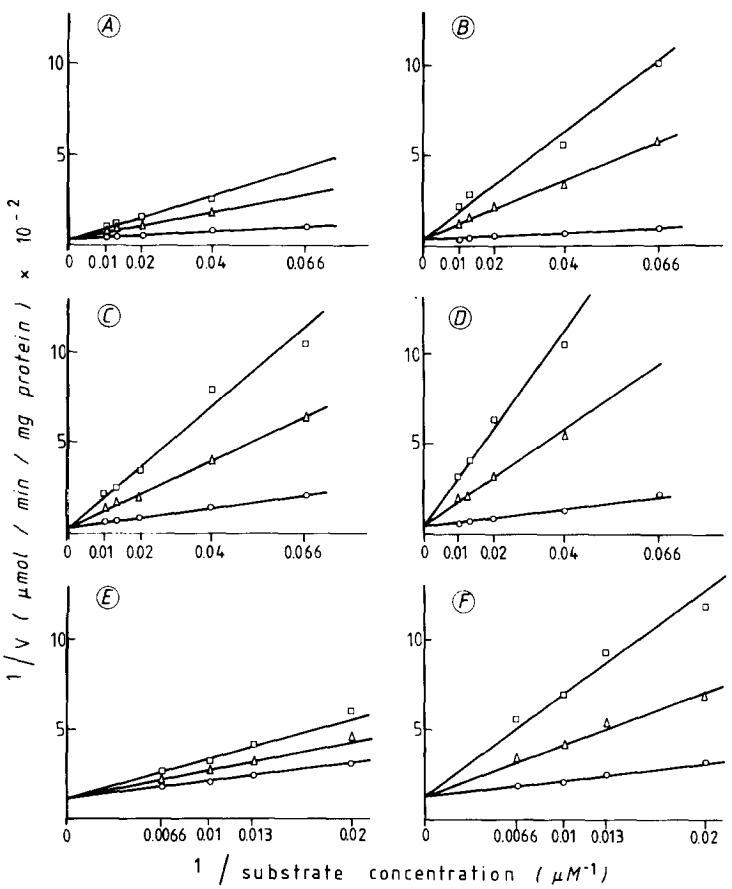

Fig. 2. Double-reciprocal plots for inhibition of beef intestine adenosine deaminase by ddDAPR and ddeDAPR with Ado (panels A and B), ddAdo (panels C and D) and araA (panels E and F) as substrates. Inhibitor concentrations : none (O); 80 µM (□) and 40 µM (Δ) for ddDAPR (panels A and C); none (O), 40 µM (□) and 20 µM (Δ) for ddDAPR (panel E); none (O), 80 µM (□) and 40 µM (Δ) for ddeDAPR (panel B); none (O), 40 µM (□) and 20 µM (Δ) for ddeDAPR (panel D); none (O), 20 µM (□) and 10 µM (Δ) for ddeDAPR (panel F).

17-20). These inhibitors consist of either an acyclic "sugar" chain (i.e. EHNA) or a modified purine skeleton (i.e. CF, ICF, DCF).

Among the purine derivatives containing an intact pentose moiety and purine ring, 1,6-dihydro-6-hydroxymethylpurine riboside (DHMPR) (21,22) is the most potent ADA inhibitor reported so far [K_i : 1.3 μM (23) and 0.48 μM (24)]. With a K_i value of 3.8-5.4 μM, ddeDAPR can also be considered as a potent inhibitor of ADA. The 2',3'-dihydrogenated derivative of ddeDAPR, ddDAPR, was 3-fold less potent as an ADA inhibitor than ddeDAPR. In both cases, inhibition was competitive with respect to Ado, ddAdo and araA as the substrate. From preincubation experiments, we could assess that ddeDAPR and ddDAPR are readily reversible inhibitors of ADA since the deamination rate of ddAdo in the presence of inhibitor did not change upon a 30 min preincubation of ADA with the inhibitor added prior to the addition of the substrate (data not shown).

Our findings that ddDAPR and even more so ddeDAPR strongly inhibit the deamination of a number of adenosine analogues including ddAdo and araA (with K_i/K_m values of ddeDAPR for deamination of ddAdo and araA being 0.05 and 0.06, respectively), make these compounds attractive candidates for combination therapy with ddAdo and araA in the treatment of retrovirus (HIV) and herpesvirus infections.

Indeed, the rapidity of deamination of adenosine analogues such as araA is a major factor limiting the chemotherapeutic effectiveness of the drug (8,9). It has been shown that on incubation with normal human erythrocytes and T-lymphocytes adenosine analogues do not form appreciable amounts of the corresponding adenylate analogues but accumulate within the cell as deaminated metabolites (25). Dalal *et al.* (26) have recently demonstrated that ddAdo is rapidly deaminated in human Molt/4F and ATH8 cells to ddIno and subsequently metabolized to the natural adenosine nucleotides. The predominant (> 98 %) radiolabelled metabolites of [^{3}H]ddAdo were ribonucleotides (i.e. ADP and ATP) (26). Since the putative active form of ddAdo is believed to be its 5'-triphosphate (ddATP) (26), attempts should be made at increasing the intracellular formation of ddATP, and hence enhancing the biological (i.e. anti-HIV) activity of ddAdo. This goal may be accomplished by simultaneous administration of ddAdo with an ADA inhibitor.

The 2,6-diaminopurine derivative ddDAPR is a potent and selective inhibitor of HIV replication (11). It is relatively non-toxic to human cells (11) and, as compared to ddAdo, a poor substrate for ADA (based on their V_{max}: Table 1). When deaminated ddDAPR generates ddGuo, a compound which is also an effective inhibitor of HIV replication *in vitro* (5). The finding that ddDAPR inhibits the deamination of ddAdo by ADA adds another therapeutic bonus to ddDAPR, as it makes it a potential candidate for combination therapy with ddAdo in the treatment of AIDS.

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