Vol. 159, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1989 Pages 61-67

THE ANTIVIRAL ACTIVITY OF 9-β-D-ARABINOFURANOSYLADENINE IS ENHANCED BY THE 2',3'-DIDEOXYRIBOSIDE, THE 2',3'-DIDEHYDRO-2',3'-DIDEOXYRIBOSIDE AND THE 3'-AZIDO-2',3'-DIDEOXYRIBOSIDE OF 2,6-DIAMINOPURINE

Jan Balzarini and Erik De Clercq

Rega Institute for Medical Research, Katholieke Universiteit Leuven,
Minderbroedersstraat 10, B-3000 Leuven, Belgium

Received January 3, 1989

SUMMARY : The 2',3'-dideoxyriboside (ddDAPR), 2',3'-didehydro-2',3'-dideoxyriboside (ddeDAPR) and 3'-azido-2',3'-dideoxyriboside (AzddDAPR) of 2,6-diaminopurine have been previously recognized as potent inhibitors of human immunodeficiency virus replication. These compounds are also potent inhibitors of adenosine deaminase and inhibit the deamination of 9-β-D-arabinofuranosyladenine (araA). ddDAPR, ddeDAPR and AzddDAPR markedly potentiate the antiviral activity of araA against herpes simplex virus type 1 (HSV-1), type 2 (HSV-2) and vaccinia virus (VV). When used at a concentration of 20 µg/ml, which had by itself no antiviral effect, ddDAPR, ddeDAPR and AzddDAPR increased the ability of araA to suppress HSV-1, HSV-2 and VV yield by several orders of magnitude. The maximum antiviral effect was obtained with the combinations of ddDAPR or ddeDAPR with araA concentrations of 1 and 10 µg/ml.

It is well documented that many adenosine analogues with potential usefulness in the chemotherapy of cancer and virus infections are subject of deamination by adenosine deaminase (EC 3.5.4.4) (ADA) (1). Most of these adenosine analogues are deaminated by ADA at lower rates than the natural substrates adenosine or 2'-deoxyadenosine (2,3). Yet, deamination continues to be a factor that complicates the therapeutic efficacy of such adenosine analogues as 9-β-D-arabinofuranosyladenine (AraA), formycin A, cordycepin (1,4).

Inhibitors of adenosine deaminase facilitate the uptake of adenosine nucleoside derivatives into cellular nucleotide pools, decrease the formation of their deaminated metabolites and enhance the chemotherapeutic efficacy of the adenosine analogues in some instances (4,5). Several highly potent inhibitors of ADA have been identified among which coformycin (6,7), isocoformycin (4), 2'-deoxycoformycin (6,8) and erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) (6,9) are the most potent (K_i values within the 10^{-9} to 10^{-12} M range).

We have recently demonstrated that 2,6-diaminopurine 2',3'-dideoxyriboside (ddDAPR) and 2,6-diaminopurine 2',3'-didehydro-2',3'-dideoxyriboside (ddeDAPR) not only are poor substrates for ADA (with V_{max} values that are 35-

0006-291X/89 $1.50

to 350-fold lower than those obtained for Ado and dAdo), but also strong inhibitors of the deamination of adenosine, 2',3'-dideoxyadenosine (ddAdo) [an inhibitor of HIV (human immunodeficiency virus)] and araA (K_i/K_m : 0.48, 0.19 and 0.23 for ddDAPR and 0.17, 0.05 and 0.06 for ddeDAPR, respectively) (10). In addition, we found that ddDAPR and to a lesser extent, ddeDAPR are potent and selective inhibitors of HIV replication (11). Also the 3'-azido derivative of ddDAPR (AzddDAPR), another 2,6-diaminopurine derivative that is deaminated to a considerably lower extent than adenosine, has proven to be a potent anti-HIV agent (12).

Because of their inhibitory effect on adenosine deaminase, ddDAPR, ddeDAPR and AzddDAPR could be expected to enhance the antiviral activity of AraA, a well-known anti-herpes drug. Such an enhancing effect on the anti-herpetic action of AraA may provide a rationale for the combined use of AraA and any of the 2,6-diaminopurine derivatives in the treatment of herpesvirus infections, and thus extend the scope of the potential therapeutic usefulness of ddDAPR, ddeDAPR and AzddDAPR.

MATERIALS AND METHODS

Compounds. Adenosine (Ado) and 9-β-D-arabinofuranosyladenine (araA) were obtained from Sigma Chemical Company (Milwaukee, Wisconsin, USA). 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 based on the conversion of Ado to ddeAdo and hydrogenation of the latter to ddAdo (13,14). The synthesis of AzddDAPR has been described previously (12).

Viruses. Herpes simplex virus type 1 (KOS strain) (HSV-1), HSV-2 (G strain), vesicular stomatitis virus (VSV) and vaccinia virus (VV) were obtained from the American Type Culture Collection (Rockville, MD).

Antiviral assays. Primary rabbit kidney (PRK) and murine L929 cells were seeded in petri dishes (6 cm diameter) and used for the assays when confluent. Then, PRK cells were infected with HSV-1, HSV-2 or VV and L929 cells with VSV at $10^{4.5}$ PFU per petri dish. After a 60 min virus adsorption period, ddDAPR, ddeDAPR or AzddDAPR at a concentration of 20 μg/ml, together with AraA (at 100, 10, 1, 0.1 or 0 μg/ml) were added. After 6 or 24 hours for VSV, 24 or 48 hours for HSV-1 and HSV-2 and 48 or 72 hours for VV, the cultures were frozen at -70°C, thawed to room temperature and centrifuged at 2000 rpm for 10 min. These supernatants were divided in aliquots and again frozen at -70°C. Then, the samples were titrated on confluent Vero monolayer cells to determine virus yield (expressed in plaque forming units/ml).

Enzymatic assay. Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) was prepared from beef intestine, and provided by Boehringer Mannheim (W.-Germany) as a suspension in ammonium sulfate solution, 3.2 mol/liter, pH 6.0. The enzyme preparation had a specific activity of ca. 200 U/mg (25°C, with adenosine as substrate). The enzyme solution was diluted in bidistilled water to a concentration of 0.1 unit/ml in the assays with Ado and to 0.5 units/ml in the assay with AraA. 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 course of deamination was recorded at 265 nm. The K_m values for Ado and AraA were determined by the Lineweaver and Burk procedure using a linear regression program. For the inhibition studies, substrate solution (i.e. Ado, AraA) was added to the sample cuvette only, and the inhibitor solution (i.e. ddDAPR, ddeDAPR, AzddDAPR) was added to both the sample and reference cuvettes. The final enzyme concentration was 0.01 unit/ml for Ado and 0.05 unit/ml for AraA. At these enzyme dilutions, no significant deamination of ddDAPR, ddeDAPR and AzddDAPR occurred within the incubation time of the assay.

RESULTS

We previously examined ddDAPR, ddeDAPR and AzddDAPR for their susceptibility to deamination by beef intestine adenosine deaminase (ADA) (10,12). Also, the K_i/K_m values of ddDAPR and ddeDAPR for ADA with Ado and AraA as the substrate were determined (10). We now found that the K_i/K_m of AzddDAPR for ADA with either Ado or AraA as substrate are comparable to those obtained before for ddeDAPR (Table 1, Fig. 1). The inhibitory effects of AzddDAPR on ADA are competitive with respect to both Ado and AraA as substrate (Fig. 1). AzddDAPR inhibited the deamination of Ado and AraA at an enzyme concentration that did not effect a significant deamination of AzddDAPR within the timespan of the experiment.

The effects of ddDAPR, ddeDAPR and AzddDAPR on the antiviral activity of AraA were evaluated in HSV-1- and HSV-2-infected PRK cell cultures (Table 2). At the concentration used (20 µg/ml) ddDAPR, ddeDAPR and AzddDAPR did not interfere with HSV-1 or HSV-2 replication. When combined with AraA (1 and 10 µg/ml), ddDAPR brought about an additional reduction of HSV-1 yield (over that achieved by AraA itself) to the amount of 1.33 log and > 2.45 log, respectively when evaluated at 24 hours after infection, and 0.7 log and 5.07 log at 48 hours after infection (Table 2). The enhancing effect of ddDAPR on the anti-HSV-1 activity of higher (100 µg/ml) or lower (0.1 µg/ml) AraA concentrations was not as prominent as that observed with 1 and 10 µg AraA per ml. The 2',3'-unsaturated derivative of ddDAPR (ddeDAPR) behaved very much like ddDAPR in its enhancing effect on the antiviral activity of AraA (Table 2). Again,

TABLE 1. K_i values of beef intestine adenosine deaminase for ddDAPR, ddeDAPR and AzddDAPR with adenosine and AraA as substrates

Substrate	K_i/K_m [a]			
	ddDAPR [b]	ddeDAPR [b]	AzddDAPR	type of inhibition
Adenosine	0.48	0.17	0.22	competitive
AraA	0.23	0.06	0.06	competitive

[a] K_m values for adenosine and araA were 29 µM and 73 µM, respectively.
[b] Data taken from ref. 10.

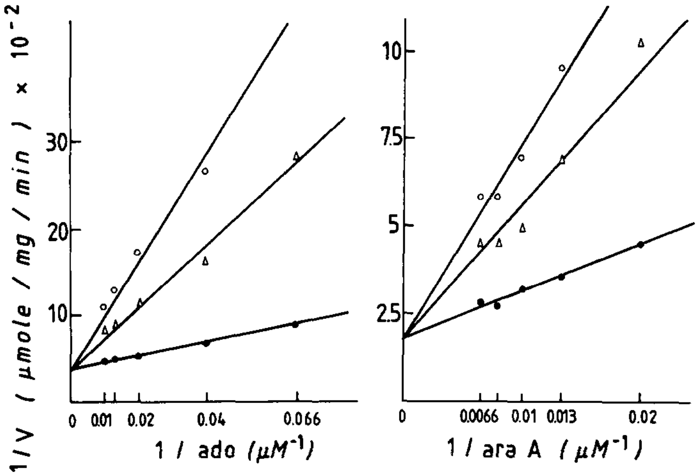

Fig. 1. Double-reciprocal plots for inhibition of beef intestine adenosine deaminase by AzddDAPR with adenosine (Ado) and 9-β-D-arabinofuranosyladenine (AraA) as substrates. Inhibitor concentrations : none (●); 30 µM (○) and 15 µM (△) for Ado; 15 µM (○) and 7.5 µM (△) for AraA.

TABLE 2. Effect of ddDAPR, ddeDAPR and AzddDAPR in combination with AraA on the yield of herpes simplex virus type 1 (KOS) and herpes simplex virus type 2 (G) in primary rabbit kidney cell cultures

Test compound (20 µg/ml)	AraA concentration (µg/ml)	HSV-1 yield^a (log$_{10}$ PFU/ml)		HSV-2 yield^a (log$_{10}$ PFU/ml)	
		24 hr	48 hr	24 h	48 h
ddDAPR	0	7.08	7.68	4.27	4.47
	0.1	6.68	7.68	4.29	4.50
	1.0	5.14	7.01	3.43	4.32
	10	< 1.30	1.60	< 1.3	2.39
	100	< 1.30	< 1.30	< 1.3	< 1.3
ddeDAPR	0	6.70	8.08	N.D.^{b}	N.D.
	0.1	6.14	7.57	N.D.	N.D.
	1.0	4.77	7.12	N.D.	N.D.
	10	< 1.30	1.60	N.D.	N.D.
	100	< 1.30	1.30	N.D.	N.D.
AzddDAPR	0	6.23	7.66	3.96	4.47
	0.1	6.11	6.59	3.31	4.31
	1.0	4.96	7.18	3.14	4.22
	10	< 1.3	2.97	< 1.3	1.6
	100	< 1.3	< 1.30	< 1.3	< 1.3
None	0	7.25	7.83	4.82	5.23
	0.1	6.97	7.77	4.64	4.3
	1.0	6.47	7.71	4.66	4.64
	10	3.75	6.53	2.14	4.09
	100	1.45	≤ 1.30	< 1.3	< 1.3

[a] As determined in Vero cells.
[b] N.D.: not determined.

the most striking enhancement was found if the concentration of AraA was 1 or 10 µg/ml. AzddDAPR also achieved an additional reduction in HSV-1 yield over that achieved by AraA alone, both at 24 hr and 48 hr, but the enhancing effect of AzddDAPR was less pronounced than that of ddDAPR or ddeDAPR. When the effects of ddDAPR and AzddDAPR were evaluated for their enhancing effect on the anti-HSV-2 activity of AraA, similar observations were made as for HSV-1. At 1 and 10 µg/ml AraA, both ddDAPR and AzddDAPR afforded an increase of anti-HSV-2 activity of AraA by 1.5 to 2.4 orders of magnitude at 24 hr and 48 h, respectively.

We also evaluated the effects of the combinations of AraA with ddDAPR, ddeDAPR or AzddDAPR on VV replication in PRK cells (Table 3). Again, the optimal concentrations of AraA at which the maximum additional reduction in virus yield was achieved by the test compounds appeared to be 1 and 10 µg/ml. With these AraA concentrations, ddDAPR afforded an additional reduction of 1.73 log and 0.84 log, respectively in the vaccinia virus yield at 72 hr. The potentiating effect of ddeDAPR on the anti-VV activity of AraA was even more striking than that of ddDAPR (Table 3). For example, the combination of ddeDAPR

TABLE 3. Effect of ddDAPR, ddeDAPR and AzddDAPR in combination with AraA on the yield of vaccinia virus in primary rabbit kidney cell cultures

Test Compound (20 µg/ml)	AraA concentration (µg/ml)	Virus yield[a] ($\log_{10}$ PFU/ml) 48 hr	72 hr
ddDAPR	0	6.95	6.26
	0.1	6.63	6.74
	1.0	4.99	5.79
	10	3.44	3.38
	100	3.69	3.01
ddeDAPR	0	7.07	7.30
	0.1	6.86	6.66
	1.0	4.17	4.35
	10	3.25	3.21
	100	3.23	3.32
AzddDAPR	0	6.79	6.28
	0.1	6.64	6.59
	1.0	5.70	6.08
	10	2.87	3.29
	100	2.64	2.74
None	0	7.31	7.00
	0.1	7.39	6.85
	1.0	6.72	6.59
	10	4.28	5.63
	100	3.83	3.05

[a] As determined in Vero cells.

with 1 μg AraA per ml resulted in an additional reduction of virus yield by 2.55 and 2.24 log at 48 and 72 hr, respectively. AzddDAPR, when combined with AraA (1 or 10 μg/ml) caused an additional reduction of 1.02 and 1.51 log in VV yield at 48 hr, and of 0.51 and 2.34 log at 72 hr, respectively.

The effects of ddDAPR and AzddDAPR on the anti-VSV activity of AraA were also evaluated in L929 cells. AraA proved only slightly active against VSV at the highest concentration tested (100 μg/ml) : at this concentration VSV yield at 6 hours and 24 hours after infection was reduced by 1.25 log and 0.67 log, respectively (data not shown). Under these experimental conditions, combination of AraA with ddDAPR or AzddDAPR did not bring about an additional reduction of VSV yield (over that achieved by AraA itself).

DISCUSSION

Several attempts have been made to increase the antiviral or antitumor activity of adenosine and cytidine analogues (i.e. AraA, AraC) by combining these drugs with compounds that inhibit their deamination. Our findings indicate that ddDAPR, ddeDAPR and AzddDAPR inhibit the deamination of AraA by adenosine deaminase and enhance the antiviral (i.e. anti-herpes, anti-vaccinia) activity of AraA. These findings may have important therapeutic implications, also because of the concomitant potent anti-HIV activity of ddDAPR and AzddDAPR (11,12). Thus, these compounds may be pursued as such for the treatment of HIV infection, and, in combination with AraA, for the treatment of HSV infection and other opportunistic virus infections in AIDS patients.

2',3'-Dideoxyadenosine (ddAdo) is currently evaluated for its therapeutic potential in AIDS patients. AzddDAPR and ddDAPR are at least as effective, if not more effective than ddAdo in inhibiting HIV replication *in vitro*, and these compounds may be considered as candidate drugs for the treatment of AIDS. In the light of our present findings, ddDAPR and AzddDAPR could also serve as useful drugs, in combination with AraA, for the treatment of those virus infections, i.e. herpes, vaccinia, that would normally be amenable to AraA therapy, and that in AIDS patients often represent a life-threatening condition.

ACKNOWLEDGMENTS

These investigations were supported by grants from the Belgian F.G.W.O. (Fonds voor Geneeskundig Wetenschappelijk Onderzoek, projects no. 3.0040.83 and no. 3.0097.87) and the Belgian G.O.A. (Geconcerteerde Onderzoeksacties, project no. 85/90-79). We thank Miette Stuyck, Anita Van Lierde and Frieda De Meyer for excellent technical assistance and Christiane Callebaut for fine editorial help.

REFERENCES

1. Agarwal, R.P., Sagar, S.M., and Parks, R.E. Jr. (1975) Biochem. Pharmacol. 24, 693-701.
2. Bloch, A., Robins, M.J., and McCarthy, J.R. Jr. (1967) J. Med. Chem. 10, 908-912.
3. Frederiksen, S. (1966) Arch. Biochem. Biophys. 113, 383-388.
4. Agarwal, R.P. (1982) Pharmac. Ther. 17, 399-429.
5. Agarwal, R.P., Cha, S., Crabtree, G.W., and Parks, R.E. Jr. (1978) In: Chemistry and Biology of Nucleosides and Nucleotides, pp. 159-197 (Harman, R.F., Robins, R.K., and Townsend, L.B., eds.). Academic Press, New York.
6. Henderson, J.F., Brox, L., Zombor, G., Hunting, D., and Lomax, C.A. (1977) Biochem. Pharmacol. 26, 1967-1972.
7. Sawa, T., Fukagawa, T., Homma, I., Takeuchi, T., and Umezawa, H. (1967) J. Antibiot. A20, 227-231.
8. Woo, P.W.K., Dion, H.W., Lange, S.M., Dahl, L.F., and Durham, L.J. (1974) J. Heterocy. Chem. 11, 641-647.
9. Schaeffer, H.J., and Schwender, C.F. (1974) J. Med. Chem. 17, 6-8.
10. Balzarini, J., Robins, M.J., Zou, R., Herdewijn, P., and De Clercq, E. (1987) Biochem. Biophys. Res. Commun. 145, 277-283.
11. Balzarini, J., Pauwels, R., Baba, M., Robins, M.J., Zou, R., Herdewijn, P., and De Clercq, E. (1987) Biochem. Biophys. Res. Commun. 145, 269-276.
12. Balzarini, J., Baba, M., Pauwels, R., Herdewijn, P., Wood, S.G., Robins, M.J., and De Clercq, E. (1988) Mol. Pharmacol. 33, 243-249.
13. Robins, M.J., Hansske, F., Low, N.H., and Park, J.I. (1984) Tetrahedron Lett. 25, 367-370.
14. Hansske, F., and Robins, M.J. (1985) Tetrahedron Lett. 26, 4295-4298.