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INITIAL STUDIES ON THE CELLULAR PHARMACOLOGY OF 2',3'-DIDEOXYINOSINE, AN INHIBITOR OF HIV INFECTIVITY

Gurpreet Ahluwalia*, David A. Cooney*, Hiroaki Mitsuya†, Arnold Fridland§, Karl P. Flora*
Zhang Hao*, Maha Dalal*, Samuel Broder† and David G. Johns*

*Developmental Therapeutics Program and †Clinical Oncology Program,
Division of Cancer Treatment, National Cancer Institute, NIH,
Bethesda, MD 20892, U.S.A.

§Division of Biochemical and Clinical Pharmacology,
St. Jude Children's Research Hospital, Memphis, TN 38101, U.S.A.

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A number of 2',3'-dideoxynucleosides inhibit the in vitro infectivity and cytopathic effect of the HIV (HTLV-III/LAV) retrovirus (1), the etiologic agent of the acquired immunodeficiency syndrome (AIDS) (2,3). Of the 2',3'-dideoxynucleosides studied to date, the purine nucleoside analogues 2',3'-dideoxyadenosine (ddAdo) and 2',3'-dideoxyinosine (ddIno) exhibit particularly favorable therapeutic ratios and appear to be equally active in terms of their anti-HIV activity in the ATH8 test system (1). In a previous study (4), we found that ddAdo was phosphorylated to yield the putative active metabolite ddATP and was also subject to rapid conversion to ddIno by the enzyme adenosine deaminase. Unlike ddAdo, however, the deamination product ddIno did not give rise to detectable amounts of its corresponding 5'-triphosphate, ddITP. The basis for the antiretroviral activity of ddIno thus remained unexplained by these earlier results, and we therefore examined its cellular pharmacology in greater detail.

To carry out these studies, [^{3}H]ddIno labeled in the 2'- and 3'-positions of the dideoxyribose moiety was prepared by means of enzymatic deamination of 2',3'-di[^{3}H-]-ddAdo (4), utilizing calf intestinal adenosine deaminase (Sigma, St. Louis, MO). In several preparations, the percentage of the radiolabel in the dideoxyribose moiety ranged from 94 to 98%, with the remaining tritium (2-6%) in the 2- and 8-positions of the purine base.

We initially compared ddIno and ddAdo for their abilities to enter cells. Human T-lymphocytes (Molt-4 and CEM) were suspended in medium that had been heat-treated in order to inactivate the endogenous adenosine deaminase present in fetal calf serum (5), and the rates of entry of [^{3}H]ddIno and [^{3}H]ddAdo were determined. Entry of ddAdo was 4- to 6-fold more rapid than that of ddIno (Fig. 1), a result compatible with the greater lipid solubility of ddAdo: [log P: -0.22 for ddAdo vs -1.168 for ddIno (1-octanol:0.1 M sodium phosphate buffer, pH 7.3)]. Deamination of ddAdo by intracellular adenosine deaminase subsequent to entry was extremely rapid, with all intracellular nucleoside ^{3}H-radioactivity cochromatographing with [^{3}H]ddIno even at the shorter time periods (0.5 to 5 min). The nucleoside transport inhibitor nitrobenzylthioinosine (NBMPR) at 20 μM did not inhibit the entry of either ddAdo or ddIno, indicating that in these T-cell lines, the interiorization of these compounds is not significantly dependent on the purine nucleoside carrier.

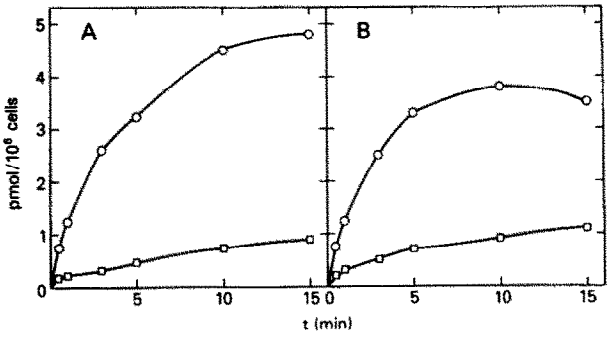

Fig. 1. Uptake of [^{3}H]ddAdo (o—o) and [^{3}H]ddIno (□—□) by CEM and Molt-4 cells. Logarithmically growing CEM (Panel A) or Molt-4 (Panel B) cells were centrifuged at 1000 g and resuspended in RPMI 1640 medium + 16% heat treated (56°, 24 hr) fetal calf serum at a density of 3.0 x 10^6 cells/ml (CEM) or 2.5 x 10^6 cells/ml (Molt-4). [^{3}H]ddAdo or [^{3}H]ddIno (1 μM; 3.4 mCi/μmol) was then added and uptake studies were carried out as previously described (6). Identity of dideoxynucleosides in the cell extracts was established by reverse-phase HPLC (7).

We next compared the intracellular metabolism of ddIno and ddAdo. When these compounds were incubated with Molt-4 cells and the resulting radiolabeled metabolites separated by ion-pairing chromatography, it was found (Fig. 2) that virtually identical metabolic profiles were generated. ddIno, like ddAdo (4), gave rise to dideoxynucleotides of the dideoxyadenylate series (ddAMP, ddADP and ddATP), and also to IMP and to adenylate ribonucleotides, the latter resulting from reutilization of the hypoxanthine released on cleavage of ddIno by purine nucleoside phosphorylase. Neither compound gave rise to ddIno anabolites other than the 5'-monophosphate, ddIMP.

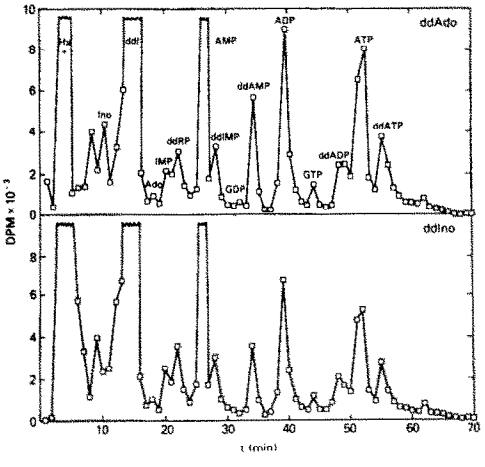

Fig. 2. Ion-pairing (C_{18}) HPLC elution profile of aqueous extracts of Molt-4 cells (12.3 x 10^6 cells) incubated for 4 hr with dideoxyribose-labeled [^{3}H]ddAdo or [^{3}H]ddIno, 5 μM, plus dAdo, 200 μM. Cell extracts were prepared as previously described (4). Extracts from triplicate samples were pooled, freeze-dried, and reconstituted in water, 250 μl. A 200-μl aliquot was subjected to ion-pairing HPLC on a C_{18} column. Solvent A: 0.02 M potassium phosphate monobasic (native pH) with 5 mM tetrabutylammonium phosphate (Aldrich Chemical Co., Milwaukee, WI); Solvent B: 100% methanol. The elution program started with a 15-min linear gradient from 100% solvent A to 90% A:10% B, followed by 25 min of slightly convex gradient to 25% B, followed by 5 and 10 min of linear gradient to 30% and 35% B, respectively, and terminated by 5 min of slightly convex gradient to 40% B. Deoxyadenosine was added to the incubation mixture to enhance formation of dideoxynucleotides (see Ref. 4).

It appears on the basis of these observations that ddIno, like ddAdo, acts as a precursor for ddATP, and presumably, therefore, exerts its pharmacological activity as an antiretroviral agent through the agency of the latter compound. We therefore attempted to define the route by which ddIno could reenter the dideoxyadenylate pathway. In our initial chromatographic studies, it appeared that, although ddIDP and ddITP could not be detected, it was possible to detect ddIMP in small but significant amounts (Fig. 2). The possibility was therefore considered that re-entry of ddIno into the ddAdo anabolic pathway occurred at the 5'-monophosphate level (i.e. ddIMP → ddAMP). Since the pathway for the generation of the physiological purine nucleotide AMP from IMP is well-defined, it was considered possible that ddIMP could also utilize this route. The initial step in the pathway (IMP + L-aspartate + GTP → adenylosuccinate + GDP) is known to be inhibited by the amino acid analogue L-alanosine (8), and we therefore first examined the effect of the latter agent on dideoxynucleotide formation. As shown in Table 1, L-alanosine, at a concentration of 50 μM, blocked entirely the formation of ddATP from ddIno and also resulted in formation of increased pools of ddIMP, effects compatible with inhibition at the mononucleotide level. Similarly, with [^{3}H]ddAdo as precursor, L-alanosine also substantially decreased the formation of ddATP, indicating the quantitative predominance of the indirect route (i.e. via ddIno) from ddAdo to ddAMP, relative to the direct phosphorylation route via 2'-deoxycytidine kinase and adenosine kinase.

Table 1. Effect of L-alanosine on formation of nucleotides from ddAdo and ddIno

	ddIMP	ddADP	ddATP
		(pmoles/10^6 cells)	
ddAdo	0.28	0.13	0.06
ddAdo + L-alanosine	1.44	0.02	0.01
ddIno	0.28	0.07	0.04
ddIno + L-alanosine	1.39	0.03	<0.01

[^{3}H]ddAdo (5 μM; 1.0 mCi/μmol) or [^{3}H]ddIno (5 μM; 1.0 mCi/μmol) was incubated with Molt-4 cells (12.3 x 10^6 cells) for 4 hr (ddAdo) or 6 hr (ddIno) in the presence or absence of L-alanosine, 50 μM. Aqueous extracts were prepared and analyzed by ion-exchange HPLC (Partisil-10 SAX) (4).

In an attempt to verify this proposed pathway, adenylosuccinate synthetase was partially purified from rabbit muscle and the substrate activity of ddIMP for this enzyme was determined. As shown in Table 2, ddIMP was utilized in this reaction at ca. 2% of the rate seen with the physiological substrate IMP.

Table 2. Comparative utilization of IMP and ddIMP by adenylosuccinate synthetase from rabbit muscle

Substrate	Adenylosuccinate lyase	Net formation of succinate intermediate (μmol/mg protein/hr)
IMP	-	2.1
	+	0.025
ddIMP (chemically synthesized)	-	0.050
	+	0.027
ddIMP (enzymatically synthesized)	-	0.049
	+	0.022

The 30-μl reaction mixture contained: 1 μCi L-[4-^{14}C]aspartate (25 μCi/μmol); 6 mM GTP, 6 mM $MgCl_2$ in 50 mM Hepes (pH 7.0); 20 μg adenylosuccinate synthetase (IMP:L-aspartate ligase [GDP], E.C. 6.3.4.4., partially purified (lyase-free from rabbit muscle); 2 mM IMP or ddIMP respectively; with and without 50 μg of yeast adenylosuccinate lyase (E.C. 4.3.2.2.). The reaction was carried out at 37° for 30 min and terminated at 95° for 1 min. Aliquots (5 μl) of the reaction mixture were assayed by paper chromatography in a solvent system of ethanol:1 M ammonium acetate: 700:300 v/v. Spots corresponding to adenylosuccinate or dideoxyadenylosuccinate (immediately adjacent to the origin) were located under UV light, circled and cut out. Radioactivity was measured by scintillation counting. The chemically synthesized ddIMP was made from ddI by a modification of the method of Dawson et al. (9); enzymatically synthesized ddIMP was produced by the deamination of commercially available ddAMP through the agency of adenylate deaminase (rabbit muscle, Sigma).

From these results, it can therefore be proposed that ddIno exerts its antiviral activity by virtue of its ability to generate ddATP, and also that ddAdo can give rise to ddATP by either of two alternate routes, i.e. directly via 5'-phosphorylation to ddAMP, and indirectly via the intermediation of ddIno (Fig. 3), with the latter route being quantitatively predominant in human T cells. The only significant difference we have detected to date between these two compounds in terms of their cellular pharmacology is the ability of ddAdo to enter T-cells more rapidly than ddIno. Because of the rapid intracellular conversion of ddAdo to ddIno, this property does not result in any difference in the subsequent metabolic disposition of the two compounds; it may, however, confer a selective advantage to ddAdo in vivo, since it could result in more rapid and efficient intracellular uptake of ddAdo than of ddIno. In general, however, these observations provide a rational basis for the empirical observation of Mitsuya and Broder (1) that ddIno and ddAdo are equivalent in terms of their anti-HIV activity, and further raise the possibility that this ability of ddIno and ddAdo to utilize a variety of pathways for activation to the 5'-triphosphate ddATP may represent an advantageous feature of these compounds.

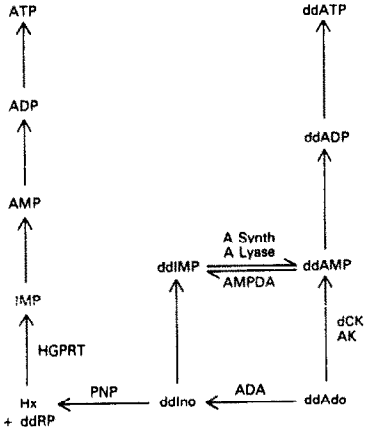

Fig. 3. Metabolism of ddAdo and ddIno. PNP: purine nucleoside phosphorylase; ADA: adenosine deaminase; HGPRT: hypoxanthine:guanine phosphoribosyltransferase; dCK: 2'-deoxycytidine kinase; AK: adenosine kinase; A Synth: adenylosuccinate synthetase; A Lyase: adenylosuccinate lyase; AMPDA: adenylate deaminase.

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