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RAPID COMMUNICATION

INITIAL STUDIES ON THE CELLULAR PHARMACOLOGY OF 2',3'-DIDEOXYADENOSINE, AN INHIBITOR OF HTLV-III INFECTIVITY

David A. Cooney*, Gurpreet Ahluwalia*, Hiroaki Mitsuya†, Arnold Fridland‡, Mark Johnson‡, Zhang Hao*, Maha Dalal*, Jan Balzarini†, 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 and related compounds inhibit the *in vitro* infectivity and cytopathic effect of the HTLV-III (HIV) retrovirus (1); this virus is the etiologic agent of the acquired immunodeficiency syndrome (AIDS) (2,3). Of the 2',3'-dideoxynucleosides studied to date, the most potent in the ATH8 test system is 2',3'-dideoxycytidine (ddCyd) (1). Superior to ddCyd in terms of therapeutic index, however, is the purine nucleoside analogue 2',3'-dideoxyadenosine (ddAdo): while the concentration of ddAdo required for maximal anti-HTLV activity in a 7-day assay is some 20-fold higher than that for ddCyd (i.e. 10 μM vs 0.5 μM), ddAdo becomes cytotoxic to ATH8 cells only at >200 μM, vs >5 μM for ddCyd (1). In this respect, ddAdo is also superior to the agent 3'-azido-3'-deoxythymidine (AZT), which has a therapeutic ratio of *ca.* 5 in the ATH8 test system (4). In view of the favorable therapeutic index of ddAdo, we have examined some of its pharmacological properties and, in particular, its ability to generate the corresponding 5'-nucleoside triphosphate (ddATP), putatively (by analogy to ddCTP and AZTTP) the active form of the drug.

Initial studies were carried out with ddAdo labeled with tritium in the 2 and 8 positions of the purine base (Moravek Biochemicals, specific activity 56 Ci/mmol, radiochemical purity > 99%). The cell line utilized was an HTLV-III/HIV-sensitive OKT4$^+$ T-cell clone (ATH8), selected on the basis of its rapid growth (in the presence of interleukin 2) and sensitivity to the cytopathic effects of the virus (1). ATH8 cells (2 x 10^7 cells) were exposed to HTLV-III at a dose of 3000 virus particles per cell; 24 hr later [2,8-^{3}H]ddAdo was added at a drug concentration of 1 μM, a level sufficient to result in detectable, although submaximal, inhibition of the cytopathic effect, infectivity and replication of the virus and without cytotoxic effect on the host cells (1). The incubation was terminated after 24 hr of drug exposure, and the methanol:acetic acid (pH 4) fraction of the cell lysate was resolved on HPLC, utilizing a radial compression column of Partisil-10 SAX with a gradient of ammonium phosphate (Fig. 1).

It was observed that ddAdo, in marked contrast to ddCyd (5), was catabolized extensively under these conditions. In a typical experiment, only a low level of the dideoxyribonucleotide ddATP (0.037 μM) was detected, and the predominant (>98%) radiolabeled metabolites were ribonucleotides coeluting with ADP and ATP (Fig. 1). This result is in general agreement with the early observation of Plunkett and Cohen (6) that ddAdo is readily converted to 2',3'-dideoxyinosine (ddIno) by adenosine deaminase, and the more recent

§Author to whom reprint requests should be sent.

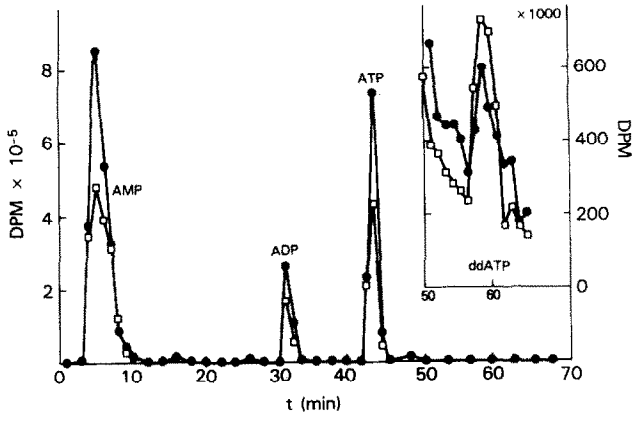

Fig. 1. Ion exchange (Partisil-10 SAX) HPLC elution profile of a 60% methanol extract of HTLV-III-infected ATH8 cells (2 x 10^7 cells) incubated for 24 hr with base-labeled [^{3}H]ddAdo (1 μM). Analyses were carried out using radial compression columns of Partisil-10 SAX equilibrated and developed with 0.01 M ammonium phosphate, pH 3.6, for 15 min followed by a linear gradient to 0.6 M ammonium phosphate, pH 3.8, over the next 25 min, and finally by 25-min isocratic elution with 0.6 M ammonium phosphate, pH 3.8. Cells were extracted with 60% methanol adjusted to pH 4 with acetic acid. Sample volume: 200 μl. Key:(●—●):^{3}H-radioactivity after incubation with [^{3}H]ddAdo, 1 μM; and (□—□): ^{3}H-radioactivity after incubation with [^{3}H]ddAdo, 1 μM, plus 2'-deoxycoformycin, 10 μM.

observation of Stoeckler et al. (7) that the deamination product ddIno is a substrate for purine nucleoside phosphorylase, both of which findings we were able to confirm with ATH8, Molt-4 and P388 cell extracts (Table 1). This reaction sequence generates hypoxanthine, from which the physiological nucleotides IMP, AMP, ADP and ATP are readily generated through well-defined purine nucleotide biosynthetic pathways. As was the case with ddCyd (5), no significant difference was noted between virus-infected and uninfected cells in ability to metabolize ddAdo (data not shown).

In an effort to block the deamination of ddAdo, we initially attempted incubation with ATH8 cells in the presence of the adenosine deaminase inhibitor 2'-deoxycoformycin, 10 μM. A slight increase (1.3-fold) in ddATP level was detected, but catabolism of ddAdo and reutilization of the purine base remained the major metabolic pathway (Fig. 1). Compatible with this finding was the observation that no significant increase in antiviral activity could be detected for ddAdo plus 2'-deoxycoformycin over that seen with ddAdo alone.

Table 1. Adenosine deaminase and purine nucleoside phosphorylase activity of ATH8, MOLT-4 and P388 cell extracts

	Adenosine deaminase activity		Purine nucleoside phosphorylase activity	
	μmol/mg/hr			
	Ado	ddAdo	Ino	ddIno
ATH8	3.3	3.7	0.41	0.01
Molt-4	2.0	2.3	0.21	0.05
P388	11.6	16.9	0.21	0.02

Exponentially growing cells were collected by centrifugation at 1000 g, resuspended in 0.05 M Tris buffer, 2 mM dithiothreitol (DTT) (pH 7.5), and ruptured by sonication. The reaction mixture was 25 μl of 12,000 g supernatant, 25 μl substrate (Ado, ddAdo, Ino or ddIno at 2 mM) and 50 μl of a 50 μM KH_2PO_4/2 mM DTT buffer, pH 7.5. Following incubation at 37° for various time periods, reactions were terminated by 1 min of heating at 95°. Product formation was determined by reverse phase HPLC, utilizing an Altex Ultrasphere ODS column (5 μ) and an isocratic mobile phase consisting of 9% acetonitrile in 50 mM ammonium acetate, pH 6.0, for the separation of Ado from Ino and of Ino from hypoxanthine, and 15% acetonitrile in 50 mM ammonium acetate, pH 6.0, for the separation of ddAdo from ddIno and of ddIno from hypoxanthine.

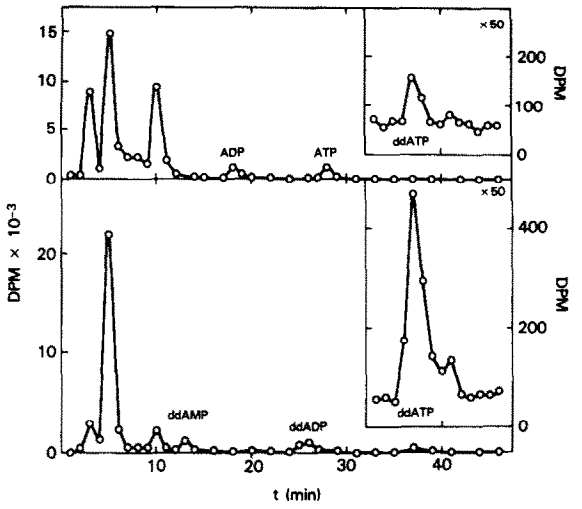

Fig. 2. Ion exchange (Partisil-10 SAX) HPLC elution profile of an aqueous extract of Molt-4 cells (6 x 10^6 cells) incubated for 4 hr with sugar-labeled [^{3}H]ddAdo, 5 μM. Incubations were carried out in RPMI 1640 medium without calf serum. Buffer A: 0.03 M ammonium phosphate, pH 4.8; buffer B: 9 parts of 0.7 M ammonium phosphate, pH 4.6, to 1 part of methanol, 100%. The following elution program was used: 5 min of buffer A, followed by 10 min of highly convex gradient to 75% buffer A:25% buffer B, followed by 15 min of slightly convex gradient to 100% buffer B, and finally by a 15-min isocratic elution with buffer B. Cells were extracted with water at 95°. Sample volume: 100 μl. Upper panel: ^{3}H-radioactivity after incubation with [^{3}H]ddAdo, 5 μM. Lower panel: ^{3}H-radioactivity after incubation with [^{3}H]ddAdo, 5 μM, plus dAdo, 100 μM. The intracellular concentrations of [^{3}H]ddATP after 4 hr of incubation with [^{3}H]ddAdo, 5 μM, were 0.068 μM (upper panel) and 0.380 μM (lower panel); these values were corrected for the percent recoveries of unlabeled ddATP carried through the same procedure.

Since, according to the pathway outlined above, only the purine moiety of ddAdo would be incorporated into adenine and guanine nucleotides, we then initiated studies with a radiolabel located predominately in the dideoxyribose portion of ddAdo, in order to better follow the metabolic fate of the ddAdo not entering the catabolic pathway. 2',3'-Dideoxyadenosin-2',3'-ene (8) was reacted with tritium gas (palladium catalyst), and the product subjected to back exchange with water, yielding radiolabeled ddAdo (specific activity 30 Ci/mmol) in which 97% of the tritium radioactivity was located in the 2'- and 3'-positions of the dideoxyribose moiety and only 3% in the 2- and 8-positions of adenine.

Utilizing this sugar-labeled material, we re-examined the cellular metabolism of ddAdo. Molt-4 cells were exposed to 2',3'-di-[^{3}H]ddAdo at a concentration of 5 μM. After 4 hr of incubation, the cells were lysed and extracted for 1 min with distilled water at 95°. After centrifugation, the supernatant fractions were subjected to HPLC on Partisil-10 SAX (Fig. 2). Using this procedure, ddATP was more readily distinguishable. Labeled ADP and ATP were still detectable but at a much lower level than with the base-labeled precursor, as would be anticipated from the metabolic route proposed above. Qualitatively, however, the distribution of radioactivity was identical except for the appearance of a new peak at 10 min (Fig. 2). Significantly, no radioactivity was detectable with the elution times of ddIDP and ddITP, indicating that the deamination step ddAdo → ddIdo is not followed by anabolic phosphorylation of the latter compound. These data indicate therefore that, while ddAdo is rapidly converted to ddIno, this step is not its pathway of pharmacological activation, but that the action of ddAdo depends rather on the small fraction of the compound which escapes deamination and instead undergoes direct anabolism to ddATP.

We next attempted to determine which nucleoside kinase (or other enzyme system) was

responsible for the activation of ddAdo, e.g. adenosine kinase (EC 2.7.1.20) and/or deoxycytidine kinase (EC 2.7.1.74) (the latter in view of its ability to phosphorylate analogous purine nucleosides such as arabinosyladenine (9)).

Utilizing deoxycytidine kinase purified 60-fold from human thymus (9), it was observed that ddAdo was indeed a substrate, with a K_m value similar to that for ddCyd (160 μM for ddAdo; 140 μM for ddCyd), but that the V_{max} for ddAdo was only half that for ddCyd (130 nmol/hr/mg vs 240 nmol/hr/mg), i.e. ddAdo, although a substrate for the enzyme, is less efficiently phosphorylated than is ddCyd. With adenosine kinase from human thymus, substrate activity for ddAdo was also detected (K_m: 50 μM), but the phosphorylation efficiency (V_{max}/K_m) was about one-tenth that of deoxycytidine kinase. No substrate activity whatsoever could be detected with adenosine kinase purified 218-fold from murine P388 cells. In alternate substrate inhibition studies, dCyd (100 μM) markedly decreased the formation of [^{3}H]ddATP from [^{3}H]ddAdo in both human (Molt-4) and murine (P-388) cells (data not shown), whereas in the presence of dAdo (100 μM), there was a 5-fold increase in the formation of ddATP (Fig. 2, lower panel). Deoxycytidine kinase would appear, therefore, to participate in the initial phosphorylation of ddAdo, while the role of adenosine kinase remains equivocal.

In summary, the cellular pharmacology of ddAdo appears to be similar to that of ddCyd in its essential feature, i.e. that ddAdo, like ddCyd, was anabolized, albeit inefficiently, to its triphosphate. In its ability to inhibit the HTLV-III reverse transcriptase, ddATP was equally as potent as ddCTP (10), an observation indicating that the lesser antiviral activity of ddAdo may be attributed to its less efficient conversion to the nucleotide. The results in this paper support this interpretation and also demonstrate that the lesser availability of ddAdo than ddCyd for anabolism is accompanied by a much greater ability of ddAdo to enter catabolic pathways, leaving a smaller fraction of drug available for direct phosphorylation, whereas ddCyd, by contrast, is almost inert to catabolism.

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