Biochemical Pharmacology Vol 35 No 13 pp 2065–2068 1986
Printed in Great Britain

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd

RAPID COMMUNICATIONS

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

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(Received 3 March 1986; accepted 3 April 1986)

A number of 2',3'-dideoxynucleosides inhibit the in vitro infectivity and cytopathic effect of HTLV-III/LAV retrovirus (1); this virus is the etiologic agent of the acquired immune deficiency syndrome (AIDS) and related diseases (2,3). Of the dideoxynucleosides studied to date, the most potent is 2',3'-dideoxycytidine (ddCyd) (1). In view of the current interest in anti-HTLV-III agents for clinical use, we have examined some of the pharmacological properties of this compound.

Of critical importance to the biological activation of nucleoside analogues, both as antiviral and as antitumor agents, is their anabolic conversion to nucleotides. We first determined, therefore, the ability of HTLV-III-infected human T-lymphoblasts to phosphorylate ddCyd. The cell line utilized was an HTLV-III/LAV-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 (2×10^7 cells) were exposed to HTLV-III/LAV at a dose of 3000 virus particles per cell; 24 hr later, [5-^{3}H]ddCyd (Moravek Biochemicals; specific activity 6 Ci/mmole, radiochemical purity >99%) was added at a drug concentration of 1 μM, a level sufficient to result in 100% inhibition of the cytopathic effect, infectivity and replication of the virus but without cytotoxic effect on the host cells (1). The incubation was terminated after 24 hr of drug exposure, and the TCA-soluble 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). Detected in the eluate were the parent nucleoside (48% of eluted radioactivity in a typical experiment, Fig. 1A), its mono-, di- and triphosphates (17%, 13% and 9%), and a peak of ^{3}H-radioactivity appearing between ddCMP and ddCDP (13% of eluted radioactivity). Reference standards were ddCDP and ddCTP (P-L Biochemicals) and ddCMP (prepared by hydrolysis of ddCTP by venom phosphodiesterase [Boehringer-Mannheim Biochemicals]). No significant qualitative or quantitative differences were detected between uninfected and HTLV-III-infected ATH8 cells in ability to phosphorylate ddCyd (Fig. 1B). However, when the physiological nucleoside dCyd (5 μM) was added together with ddCyd, phosphorylation was blocked (Fig. 1C), a result compatible with the observation that dCyd reverses the anti-HTLV-III effect of ddCyd (H. Mitsuya and S. Broder, unpublished).

The unusual antiviral specificity of ddCyd prompted us to determine whether the ability to convert ddCyd to its phosphorylated anabolites was solely a property of host cells for the virus (e.g. activated T4 cells). Studies of ddCyd anabolism were therefore carried out

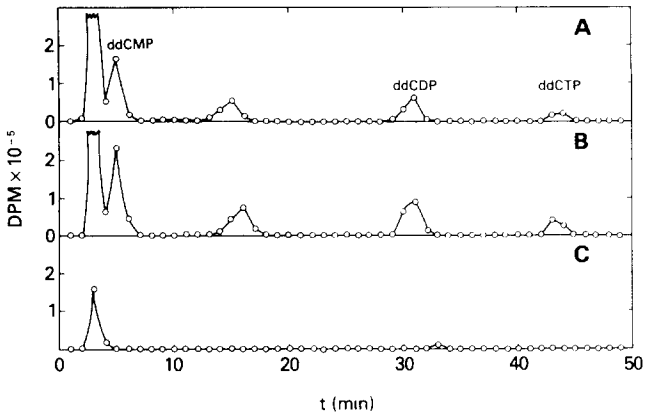

Fig. 1. Ion exchange (Partisil SAX) HPLC elution profile of a 10% TCA extract of ATH8 cells (2 x 10^7 cells) incubated for 24 hr with [^{3}H]ddCyd (1 μM). These and subsequent analyses were carried out using radial compression columns of Partisil-SAX equilibrated and developed with 0.01 M ammonium phosphate, 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 a 5 min isocratic elution with 0.6 M ammonium phosphate, pH 3.8. TCA extracts were neutralized with tri-n-octylamine in freon before analysis. Sample volume: 100 μl. Elution times: ddCyd, 3 min; ddCMP, 4-6 min; ddCDP, 30-34 min; ddCTP, 42-47 min. (A) ATH8 cells infected with HTLV-III (3000 virus particles/cell) 24 hr before addition of ddCyd. (B) ATH8 cells uninfected with HTLV-III. (C) ATH8 cells exposed simultaneously to [^{3}H]ddCyd (1 μM) and unlabeled dCyd (5 μM).

with a panel of human, murine and caprine cell lines of both lymphoid and non-lymphoid origin. As shown in Table 1, the ability to activate ddCyd was demonstrated in all lines examined, except for P388/AAC, a cell line deficient in deoxycytidine kinase. The evidence to date is therefore compatible with the interpretation that the unusual specificity of ddCyd does not lie in any unusual ability of ATH8 cells to activate the drug.

The identity of the unknown ^{3}H-labeled anabolite eluting between ddCMP and ddCDP was examined next. This anabolite was resistant to alkaline phosphatase (Sigma Chemical Co.) under conditions where the di- and triphosphates were readily hydrolyzed back to ddCyd, but was a substrate for venom phosphodiesterase (Boehringer-Mannheim Biochemicals), yielding a single peak of ^{3}H-labeled ddCMP. On these grounds, and the known ability of cytidine and arabinosylcytosine to form choline adducts (4), we incubated [^{3}H]ddCyd (50 μM) and [^{14}C]-choline (100 μM; specific activity 5 mCi/mmole) with ATH8 cells for 24 hr and examined the TCA-soluble fraction of the cell lysate as previously described. A doubly-labeled (^{3}H and ^{14}C) peak was detected at the elution position of the unidentified metabolite; this peak was not formed when ddCyd was omitted (Fig. 2). On these grounds and the susceptibility of the compound to venom phosphodiesterase but not alkaline phosphatase, an identification as ddCDP-choline is proposed.

Of particular pharmacological importance with cytidine analogues such as arabinosylcytosine is their sensitivity to enzymatic deamination *in vivo*. Structure-activity studies with cytosine nucleosides have indicated that, while a 2'-hydroxyl is not required, a 3'-hydroxyl in the "down" position is essential for pyrimidine nucleoside deaminase activity (5). To determine whether this rule applies to ddCyd, we carried out studies in which ddCyd was incubated with partially purified cytidine deaminase from mouse kidney (6) for periods up to 24 hr. No evidence for deamination was obtained by spectrophotometric assay (6), under conditions where 2'-deoxycytidine and arabinosylcytosine were rapidly deaminated. Similarly, no evidence for phosphorolysis of ddCyd to cytosine and dideoxyribose-1-phosphate

Table 1. Anabolism of [^{3}H]ddCyd by human (H), murine (M) and caprine (C) cell lines

Cell line	Cell type	pmoles/10^6 cells ddCyd	ddCMP	ddCDPCh	ddCDP	ddCTP
ATH8 (H)	OKT4$^+$ T-cells	6.40	2.10	1.10	1.20	0.50
NCI-H125 (H)	Adenosquamous Ca	2.40	6.37	7.81	7.84	4.48
NCI-H322 (H)	Bronchiolo-alveolar Ca	1.20	0.88	0.32	1.50	0.37
NCI-H358 (H)	Bronchiolo-alveolar Ca	1.38	3.32	0.29	4.80	0.66
NCI-H460 (H)	Large cell Ca	0.52	0.03	<0.01	0.07	0.03
-	Lymphocytes (H) (PHA)	1.71	0.32	0.22	0.08	0.32
Tahr (C)	Ovarian	1.40	<0.01	0.06	0.26	0.11
P388 (M)	Macrophage	0.60	0.08	0.04	0.30	0.17
P388/AAC (M)	Macrophage	1.45	<0.01	<0.01	<0.01	<0.01
L1210 (M)	Lymphoblast	1.38	0.04	0.04	0.16	0.26
3T3 (M)	Fibroblast	0.87	0.02	0.01	0.09	0.04

After incubation with [^{3}H]ddCyd (1 μM) for 24 hr, cells were extracted with 10% TCA; extracts were neutralized with tri-n-octylamine in freon and were analyzed as described in the legend for Fig. 1. Values shown are the means of replicate analysis of duplicate cultures. PHA = phytohemagglutinin-stimulated. No cytotoxicity was observed with 1 μM ddCyd.

could be obtained, a result in keeping with the known resistance of nucleosides of the cytidine family to attack by pyrimidine nucleoside phosphorylase (7).

As previously indicated, the anti-HTLV-III effect of ddCyd was reversed by dCyd, an observation readily explained by effective competition of dCyd for deoxycytidine kinase, resulting in inability to form ddCMP (Fig. 1C). To determine whether the reversal by dCyd could also be attributed in part to competition for entry into the cell, studies were carried out on the uptake of [^{3}H]ddCyd by both human and murine (Fig. 3) lymphoblasts. 2'-dCyd at 50-fold excess (50 μM) was an inefficient inhibitor of the uptake of ddCyd, although nitrobenzylthiosine (NBMPR) at 20 μM was an effective inhibitor, indicating that ddCyd transport is dependent, at least in part, on the nucleoside carrier.

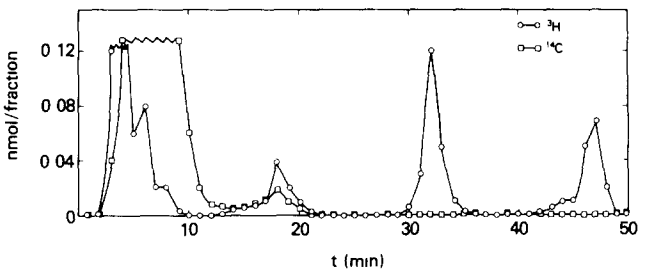

Fig. 2. Ion exchange (Partisil-10 SAX) HPLC elution profile of a 10% TCA extract of ATH8 cells. ATH8 cells (2 x 10^7 cells) were incubated for 24 hr with [^{14}C]choline (100 μM) in the presence or absence of 50 μM [^{3}H]ddCyd. Key: (□—□) [^{14}C]radioactivity in the presence of ddCyd; and (○—○) [^{3}H]radioactivity in the presence of ddCyd. In the absence of ddCyd, no [^{14}C]radioactivity was detected after fraction 14. One-minute fractions were collected. No correction has been made for the effect of endogenous cellular choline pools on the specific radioactivity of the [^{14}C]choline used.

Anabolic phosphorylation of other dideoxynucleosides (ddThd, ddAdo and ddGuo) has been studied in mammalian cell lines, but comparable studies on the metabolic activation of ddCyd have not been carried out previously, due to unavailability of the radiolabeled compound (8). Such studies with ddCyd have taken on added importance, however, in view of its unusually high potency as an anti-HTLV-III agent in vitro (compared to other dideoxynucleosides), and its rapid development toward clinical trial. In other retroviral test systems, the activity of the dideoxynucleoside triphosphates has been attributed to sensitivity to inhibition of the retroviral reverse transcriptase and the relative resistance to such inhibition of cellu-

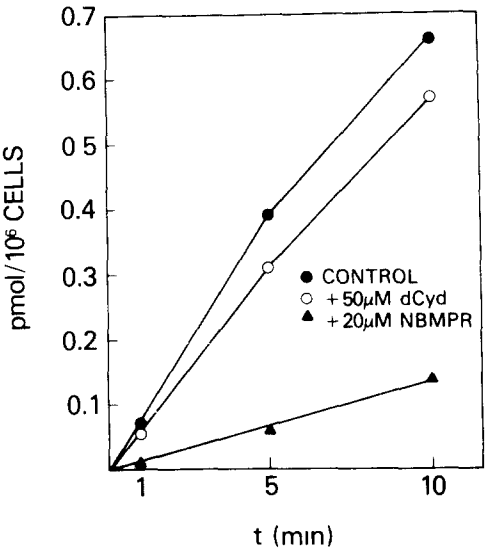

Fig. 3. Influence of dCyd and NBMPR on the uptake of [^{3}H]ddCyd by P388 cells. Logarithmically growing P388 cells were centrifuged at 1000 g and resuspended in RPMI 1630 medium + 16% fetal bovine serum at a density of 4.5 x 10^6 cells/ml, in the presence of dCyd (50 μM), NBMPR (20 μM) or saline. The cell suspensions (500 μl) were then incubated at 37° with [^{3}H]ddCyd (1 μM) for the indicated time periods, and layered over 700 μl of Versilube oil. To terminate the incubation, the cells were centrifuged through the oil layer and immediately frozen on dry ice. With the pellet still frozen, the Eppendorf tube tip was cut and the cell pellet digested with 1 ml of 1 N NaOH overnight, neutralized with equimolar HCl, and counted for radioactivity.

lar DNA polymerase α (8). The present studies are compatible with the supposition that this interpretation applies to HTLV-III also and do not support the alternate hypothesis that HTLV-III-infected T-lymphocytes are more efficient than other cells in generating the dideoxynucleoside triphosphate inhibitor; indeed, except for one cell line known to lack deoxycytidine kinase, all cell lines examined, both human and murine, were able to convert ddCyd to its active form. Whether the previously undescribed anabolite ddCDP-choline plays any primary or secondary role in the pharmacological activity of ddCyd is currently under investigation.

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