Journal of Chromatography, 133 (1977) 335–343

CHROM. 9767

ANALYSIS BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY OF 9-β-D-ARABINOFURANOSYLADENINE 5′-TRIPHOSPHATE LEVELS IN MURINE LEUKEMIA CELLS

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(Received August 25th, 1976)

SUMMARY

The metabolism of 9-β-D-arabinofuranosyladenine (AraA) to arabinofuranosyladenine 5′-triphosphate (AraATP), an inhibitor of DNA synthesis, in mouse leukemia cells was examined by means of high-pressure liquid chromatography. AraATP was separated from naturally occurring nucleotides in acid-soluble extracts and quantitative measurements of AraATP levels were made. A potent inhibitor of adenosine deaminase (2′-deoxycoformycin; co-vidarabine), when used in combination with AraA in the treatment of leukemia-bearing mice, increased the formation of AraATP in mouse leukemia cells four- to five-fold over that obtained by treatment with AraA alone. By means of high-pressure liquid chromatography the half-life of AraATP in tumor cells could be measured. Results of such studies may be of value in planning chemotherapy regimens.

INTRODUCTION

Arabinofuranosylpyrimidines and -purines have exhibited interesting biological activities[1,2]. Arabinosylcytosine is of particular interest because of its effectiveness in the treatment of acute myeloblastic leukemia in man and arabinosyladenine (AraA) because of its antiviral activity in experimental animals and in humans[3,4].

AraA was shown to inhibit DNA synthesis in bacteria[5], in mouse fibroblasts in cell culture[6], and in experimental tumors in mice[7,8]. The nucleoside is phosphorylated, primarily to the 5′-triphosphate, or is deaminated and excreted as arabinosylhypoxanthine[7,9]. AraATP is an inhibitor of ribonucleotide reductase[10,11] and of DNA polymerase[11–14]; the latter effect is considered to be responsible for limiting DNA synthesis[13]. Several investigators have suggested that the biological activity of AraA may be enhanced by inhibition of adenosine deaminase[15–17] and others have shown that the antitumor activity of AraA is increased when this agent is used in combination with inhibitors of adenosine deaminase[18–20]. Schabel *et al.*[21] have confirmed and extended the observations of Plunkett and Cohen[19] and of LePage *et al.*[20] and have obtained cures in mice bearing L1210 and P388 mouse leukemias by treatment with a combination of AraA and an adenosine deaminase inhibitor.

Studies in this laboratory have shown that AraATP can be separated from ATP and other ribonucleotides by means of high-pressure liquid chromatography (HPLC). This technique has proven useful in making quantitative measurements of levels of AraATP in extracts from tumor cells treated with AraA. Effects of inhibition of adenosine deaminase on levels of AraATP in tumor cells were also studied. Results of these studies and the technique by which the analysis was made form the basis of this communication. A preliminary report of aspects of this work has been presented[22].

EXPERIMENTAL

Chemicals

Purine and pyrimidine ribonucleotides were obtained from P-L Biochemicals (Milwaukee, Wisc., U.S.A.); 9-β-D-arabinofuranosyladenine (AraA; NSC-40421) was obtained from the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health (Bethesda, Md., U.S.A.), AraAMP, AraADP, and AraATP (5'-mono-, di- and triphosphates of AraA) were purchased from Terra-Marine Bioresearch (LaJolla, Calif., U.S.A.) or from P-L Biochemicals; [^{3}H]AraA (11 Ci/mmole) and liquid scintillation counting fluid (Aquasol) were purchased from New England Nuclear (Boston, Mass., U.S.A.). (R)-3-(2-Deoxy-β-D-*erythro*-pentofuranosyl)-3,6,7,8-tetrahydroimidazo[4,5-*d*][1,3]diazepin-8-ol (2'-deoxycoformycin; co-vidarabine; PD-ADI; NSC-218321) was obtained from Dr. Harold E. Machamer of Parke-Davis (Detroit, Mich., U.S.A.) and from the Drug Development Branch, Division of Cancer Treatment. Aqueous buffers were prepared from reagent-grade ammonium dihydrogen phosphate (J. T. Baker, Phillipsburgh, N.J., U.S.A.); buffers were adjusted to the desired pH with 1 *N* HCl.

Apparatus and analytical procedures

A Waters Associates' ALC 202 high-pressure liquid chromatograph equipped with two Model 6000 pumps, a Model 660 gradient programmer, and a Partisil-10 SAX column (25 cm × 4.6 mm, Whatman, Clifton, N.J., U.S.A.) was used to separate nucleotides. Samples (10–100 μl) were introduced by means of the Model U6K LC-injection system (Waters Assoc., Milford, Mass., U.S.A.). Resolution of the nucleotides was achieved at room temperature using a linear gradient (40 min) from 5 m*M* (pH 2.8) to 750 m*M* ammonium dihydrogen phosphate (pH 3.7) at a flow-rate of 2 ml/min (1200 p.s.i.). The eluting materials were detected by UV absorption at 254 nm and the UV signals were quantitated by a Hewlett-Packard Model 3380A digital electronic integrator; by this means concentrations of nucleotides were determined.

Standardization and quantitation

Standard solutions of the twelve ribonucleotides were prepared such that the final concentration was about 1 m*M*. The performance of the apparatus and anion-exchange column was checked at least twice a week by loading 10 μl of the above mixture of nucleotides on the column and running the linear gradient described

above. AMP, GMP, UMP, and CMP were used as standards to correlate the concentration of nucleotides, measured at 254 nm, with the area under the peaks. Solutions of AraAMP (0.2 m*M*) were prepared and the concentration of each nucleotide was determined in a Beckman DU spectrophotometer. Known volumes of each nucleotide solution were used to calibrate the system[23].

Metabolism of [^{3}H]AraA

The metabolism of [^{3}H]AraA to nucleotides was examined by collecting fractions from the column eluate directly into vials (Gilson Model TDCE fraction collector) and quantitatively determining radioactivity in each 1- or 2-ml fraction by addition of Aquasol to a final volume of 10 ml and measuring radioactivity in a Packard liquid scintillation spectrometer.

Preparation of cell extracts

L1210/0 cells (10^5 cells/mouse) were implanted intraperitoneally (i.p.) in BDF1 female mice. Six days later, groups of five mice were treated i.p. with AraA or with AraA + ADI; dose levels and time intervals between treatment and cell harvest are indicated in tables and figures. Mice were sacrificed at various time intervals after treatment and the ascites tumor cells were removed with a heparinized syringe. Tumor cells were separated from ascites fluid by centrifugation and were washed with cold (4°) Earle's solution[24]. Cold Tris-buffered isotonic ammonium chloride[25] was used to lyse red blood cells and the tumor cells so treated were washed twice with cold Earle's solution before they were extracted with cold 0.5 *N* perchloric acid (2 ml per 10^9 cells). After centrifugation the supernatant solution was removed and neutralized

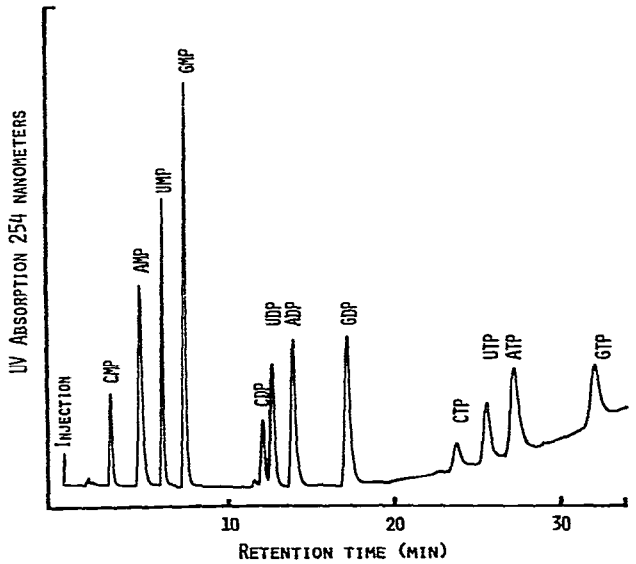

Fig. 1. A 5-μl aliquot of a standard aqueous solution of purine and pyrimidine ribonucleotides (1 m*M*) was separated, as shown above, on a Whatman Partisil-10 SAX column using a linear ammonium dihydrogen phosphate gradient (see Experimental). The UV detector-integrator was attenuated so that $A_{254} = 0.128$ (full-scale deflection).

with cold 5 *N* potassium hydroxide; potassium perchlorate was removed by centrifugation. The neutralized extract was evaporated to dryness under reduced pressure and the residue was dissolved in water (extract from 10^9 cells per ml); 10–50 μl samples of extract were used for analysis by HPLC.

RESULTS AND DISCUSSION

Analysis of a standard mixture of nucleotides

A standard aqueous solution of purine and pyrimidine ribonucleoside 5'-mono-, di- and triphosphates was separated in twelve components, as shown in Fig.

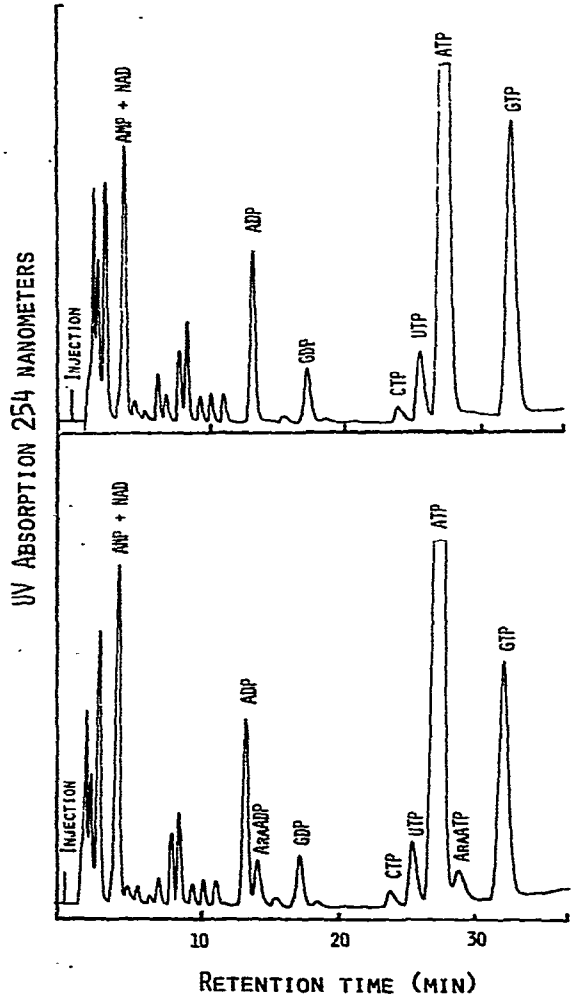

Fig. 2. HPLC analysis of a cold perchloric acid extract of L1210 mouse leukemia cells. Preparation of the extract is described in Experimental. A 10-μl aliquot of control extract (10^9 cells/ml) gave the pattern shown in the upper panel. Reference known samples of AraAMP, AraADP, and AraATP were added to the control extract to a final concentration of 0.1 m*M* with the result shown in the lower panel. AraADP and AraATP were separated from the corresponding adenine nucleotides.

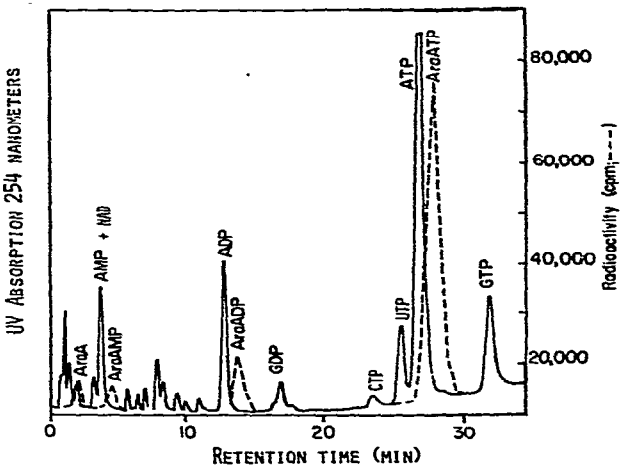

Fig. 3. Metabolism of [^{3}H]AraA by L1210 leukemia cells *in vivo*. PD-ADI (0.25 mg/kg) and [^{3}H]AraA (100 mg/kg; 25 μCi/mouse) were administered i.p. Ascites tumor cells were harvested 2 h later and perchloric acid extracts were analyzed by HPLC. One-minute fractions were collected and radioactivity was determined by liquid scintillation spectrometry.

1. The nucleoside 5′-monophosphates were eluted as sharply defined peaks within the first 10 min; broadening of peaks occurred with increased retention time of the nucleoside 5′-di- and triphosphates.

Analysis of AraATP in cell extracts

HPLC analysis of cold perchloric acid extracts of mouse leukemia cells gave satisfactory results, as shown in Fig. 2. Not all of the peaks eluted during the first 12 min have been identified; among the components known to be eluted early, in addition to the nucleoside 5′-monophosphates, are CDPcholine, CDPethanolamine, and

TABLE I

DISTRIBUTION OF RADIOACTIVITY IN L1210 LEUKEMIA CELLS AFTER TREATMENT *IN VIVO* WITH AN ADENOSINE DEAMINASE INHIBITOR IN COMBINATION WITH [^{3}H]AraA

For conditions of HPLC analysis, see the legend to Fig. 3 and Experimental.

Purine derivative	*Retention time (min)*	*L1210*	
		dpm/10^7 cells	*% of total*
AraA	2.2	6,545	7
AMP	3.7		
AraAMP	4.5	5,270	6
ADP	12.8		
AraADP	14.0	11,560	13
ATP	27.2		
AraATP	28.7	65,700	74
GTP	32.3		
	Total dpm	89,075	

UDPglucose. Adenine, guanine, cytosine, uracil, and their nucleosides were not retained on the anion-exchange column. The nucleoside 5′-di- and tri-phosphates in the leukemia cell extracts were clearly defined and were well separated. Known concentrations of AraAMP, AraADP, and AraATP were added to PCA extracts from untreated control cells. The arabinofuranosyl derivatives were retained longer than were the ribofuranosyl derivatives and could be separated from the corresponding adenine ribonucleotides.

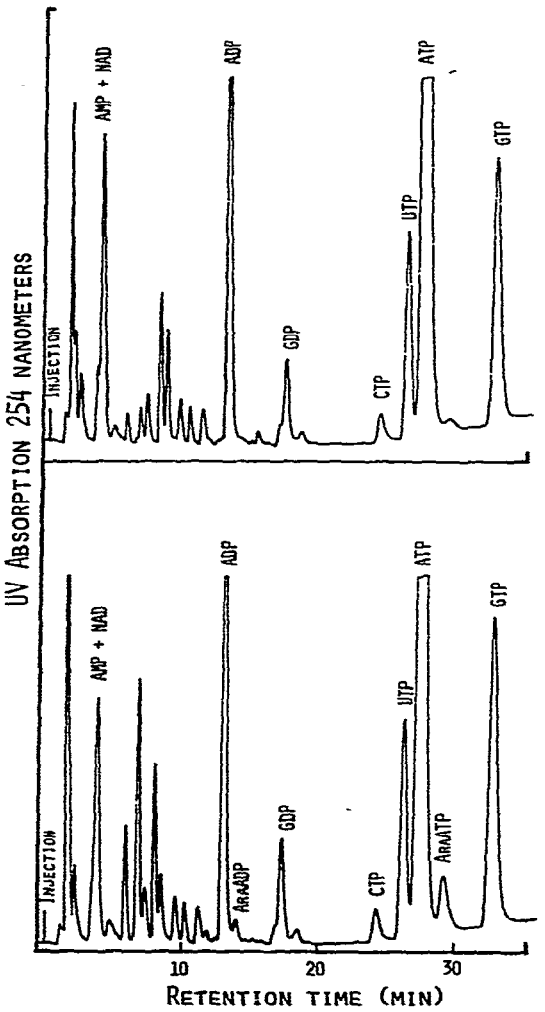

Fig. 4. Effect of an inhibitor of adenosine deaminase on the level of AraATP in L1210 cells. Mice bearing L1210 cells were treated with AraA alone (80 mg/kg) or with PD-ADI (0.2 mg/kg) + AraA. Cells were harvested 1 h later; cold perchloric acid extracts were prepared and analyzed by HPLC. The upper panel presents the analysis of extracts from cells treated with AraA alone. The lower panel shows results of treatment with the adenosine deaminase inhibitor in combination with AraA. The level of AraATP was quantitatively determined by means of an electronic digital integrator (see Table II).

Metabolism of [^{3}H]AraA in L1210 cells

The metabolism of [^{3}H]AraA in mouse leukemia cells was examined by means of HPLC with the results shown in Fig. 3. AraATP was the major metabolite of AraA in cells from animals that were simultaneously treated with AraA and 2'-deoxycoformycin (PD-ADI). Of the total radioactivity in the acid-soluble fraction 74% was present as AraATP (Table I). Arabinofuranosylhypoxanthine (AraH), if present, would be eluted with AraA; this component (AraA + AraH) constituted only 7% of the total radioactivity in the acid-soluble extract from leukemia cells harvested 2 h after the animals had been treated.

Enhancement of AraATP formation by an inhibitor of adenosine deaminase

The effect of the adenosine deaminase inhibitor on intracellular levels of AraATP is illustrated by the results presented in Fig. 4. L1210 leukemia cells that were treated *in vivo* with AraA alone or with PD-ADI + AraA, at the doses indicated, were harvested 1 h later and cell extracts were prepared for analysis. AraATP was near the lower limit of detection in animals treated with AraA alone but was elevated as much as fourfold in animals treated with the combination of agents (Table II). In animals treated with both agents, the AraATP concentration decreased with time until, 6 h after treatment, it was approximately the same as it was in cells from animals treated with AraA alone. There were no marked changes in the intracellular levels of adenine and guanine nucleotides during the time course of the experiment. Some increase in the intracellular pools of CTP and UTP were noted in extracts from cells treated with PD-ADI + AraA. PD-ADI alone (0.2 mg/kg; 1.0 mg/kg) had no significant effect on purine and pyrimidine ribonucleotide pools (data not presented).

TABLE II

NUCLEOTIDE POOLS IN L1210 LEUKEMIA CELLS TREATED *IN VIVO* WITH AraA OR WITH PD-ADI + AraA

See legend to Fig. 4.

Treatment	*Time after treatment (h)*	*Nanomoles nucleotide per 10^9 L1210 cells*							
		ADP	*AraADP*	*ATP*	*AraATP*	*GDP*	*GTP*	*CTP*	*UTP*
AraA (80 mg/kg)	1	312	—	1483	18	81	328	58	303
	2	310	8	1931	23	78	416	67	403
	4	225	4	1441	10	59	305	54	289
	6	275	5	1501	11	76	317	68	372
AraA (80 mg/kg)	1	320	18	1303	79	101	403	71	335
+	2	228	8	1423	47	73	439	104	432
PD-ADI (0.2 mg/kg)	4	313	—	1479	22	93	418	100	429
	6	335	—	1530	14	86	379	101	423

Results of more extensive experiments on the increase in AraATP formation in animals treated with PD-ADI are summarized in Fig. 5. In these experiments, a higher dose of AraA, 200 mg/kg, was used. This dose, in combination with PD-ADI (0.2 mg/kg), approaches the LD_{10} for a single treatment with the combination of agents[26]. AraATP levels exceeded 200 nmoles/10^9 cells 30 min after treatment with

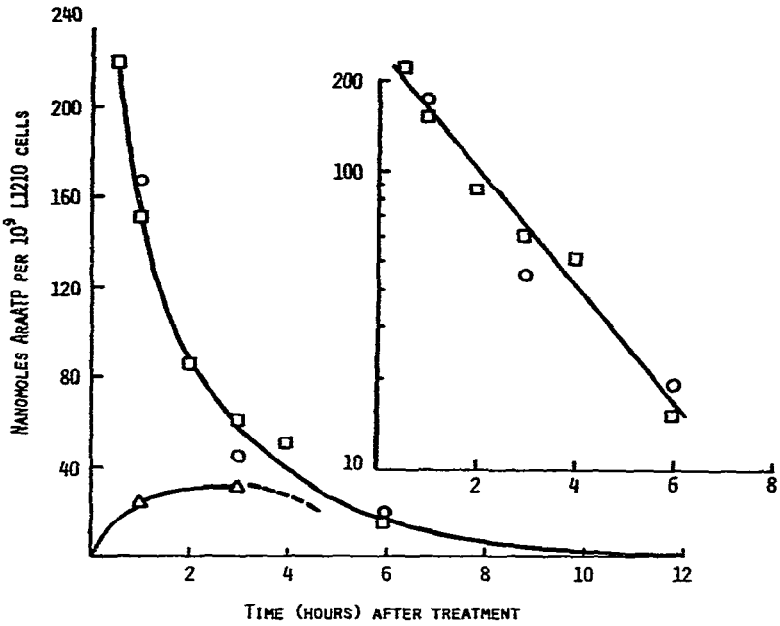

Fig. 5. AraATP levels in L1210 cells treated with AraA alone (200 mg/kg; △) or with AraA + PD-ADI (0.2 mg/kg; ○, □). The inset shows the change in AraATP concentration over a 6-h period.

AraA + PD-ADI, more than five times the level attained in cells treated with AraA alone. The concentration of AraATP fell rapidly ($t\frac{1}{2} = 2$ h) and AraATP was not detectable 12 h after treatment.

It is known from the work of LePage *et al.*[20] that low doses of 2′-deoxycoformycin (PD-ADI) almost completely inhibited adenosine deaminase activity in L1210 cells for a period of 6 h; significant inhibition of enzyme activity was observed 24 h after treatment with this agent. This observation has been confirmed in our laboratory. Thus, the rapid decline in AraATP levels within 6 h after treatment with AraA + PD-ADI is not related to adenosine deaminase activity. We have observed that in order to maintain high intracellular levels of AraATP in L1210 cells *in vivo*, even in animals treated with PD-ADI, it is necessary to treat the leukemia-bearing animals with AraA at frequent intervals. LePage *et al.*[20] and Schabel *et al.*[21] found it necessary, after inhibiting adenosine deaminase, to treat leukemic mice with AraA every 3 h over a 24-h period in order to achieve success in the chemotherapy of mouse leukemia.

By means of HPLC, quantitative measurements of AraATP levels in tumor cells and in normal cells can be made. Such data are of value in designing chemotherapy treatment schedules since inhibitory effects of AraATP on DNA synthesis can be correlated with intracellular levels of AraATP[22].

ACKNOWLEDGEMENTS

The authors are indebted to Miss Tommie Lou Barker, Mrs. Mary Elise Trader and Mr. Maxie Witt for their able assistance and cooperation in the studies

with mouse leukemia and to Dr. Frank M. Schabel, Jr., for helpful discussions of aspects of the work. This work was supported by Contract N01-CM-43784 from the Division of Cancer Treatment, National Cancer Institute, NIH, DHEW.

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