Some Inhibitory Effects of 9-β-D-Xylofuranosyladenine, an Adenosine Analog, on Nucleotide Metabolism in Ascites Tumor Cells

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SUMMARY

Xylosyl adenine (XA) was found to prolong the survival time of mice bearing TA3 or Ehrlich ascites cell tumors. Experiments with either TA3 or Ehrlich cells demonstrated that XA markedly inhibited the incorporation of ^{14}C-labeled adenine or glycine into both RNA and DNA.

XA was relatively rapidly converted *in vivo* and *in vitro* to the triphosphate (XATP).

XA was as effective as adenosine as a feedback inhibitor of purine synthesis.

XATP was found to be profoundly inhibitory to the formation of 5-phosphoribosyl-1-pyrophosphate (PRPP). This effect on the PRPP supply appeared to be the most likely explanation for the inhibitory effects on RNA and DNA synthesis, and for the inhibition of cell growth.

INTRODUCTION

The value of many agents possessing tumor inhibitory properties is greatly limited by the development of resistance to these drugs. It is well established that many analogs of nucleic acid purines become active by being converted to the nucleotide form (1, 2). In a number of instances, development of resistance is associated with the loss or decreased activity of the enzymes responsible for converting the analogs to their nucleotide forms (2–4). The potential utility of nucleoside analogs in producing tumor inhibitory effects with cells resistant to purine analogs has been suggested (5).

Over several years, the attention of this laboratory has been directed toward the accumulation of a spectrum of carcinostatic agents with widely differing modes of action. We have recently been studying the antitumor activity of nucleoside analogs modified by substitutions in the sugar moiety (6–9). We have reported on the metabolic effects of 9-β-arabinofuranosyl adenine and have demonstrated that this compound is an effective inhibitor of the growth of several murine ascites tumors (6, 7). In an extension of this search for potential antimetabolites we have studied the adenosine analog 9-β-D-xylofuranosyl adenine (XA)[2,3] (9). We have measured the tumor-inhibitory potency of this analog and have investigated some of its biochemical properties, both *in vivo* and *in vitro*.

MATERIALS AND METHODS

XA was obtained from Drs. Leon Goodman and W. Lee of this Institute. TA3,

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[2] Abbreviations used are XA, 9-β-D-xylofuranosyladenine; FGAR, α-*N*-formylglycinamide ribonucleotide; XATP, the 5'-triphosphate of xylofuranosyladenine.

[3] D. B. Ellis and G. A. LePage, unpublished.

Ehrlich, and Mecca ascites tumors were grown in female BAF1, Swiss, and AKD2F1 mice, respectively. Tumor cells were used for biochemical studies 6 days after inoculation. The sensitivity of transplanted ascites tumor cells to XA was determined by measuring both the prolongation of survival time and the decrease in volume of tumor cells afforded by the drug treatments, with previously described techniques (4, 6, 7). For *in vivo* studies, XA was dissolved in saline.

Measurement of the degree of inhibition of the metabolic pathways involved in the biosynthesis of nucleotides was performed by giving a single intraperitoneal injection of 25 mg/kg of drug to mice bearing 6-day-old ascites tumor cells. At indicated time intervals after this dose, each mouse was injected intraperitoneally with either 75 μg of adenine-8-^{14}C (1.6 μC/μmole) or 200 μg glycine-2-^{14}C (1 μC/μmole). One hour after administration of the labeled compounds the mice were sacrificed, the cells were removed and separated by centrifugation, and the specific activity of the acid-soluble adenine and the nucleic acid purines was determined (10).

In vitro studies were performed with cells washed essentially free of erythrocytes. Incubations were carried out in modified Robinson's medium (11) or in Krebs-Ringer phosphate medium pH 7.4. After incubations, cells were washed once with 8 ml of the appropriate ice cold medium and then extracted with 5 ml cold 5% trichloroacetic acid (TCA). A further extraction with 3 ml 5% TCA was carried out and the combination of the two extracts is termed the acid-soluble fraction. Under certain conditions cold 0.2 M perchloric acid (PCA) was used in place of TCA.

Orthophosphate was determined using Bartlett's modification (12) of the method of Fiske and Subbarow (13). Acid-labile phosphate was measured after hydrolysis in 1 N H_2SO_4 for 7 min, and total phosphate after ashing with H_2SO_4—HNO_3. In studies on the effect of XA on nucleotide and protein metabolism *in vitro*, conventional methods were employed (14, 15).

Feedback inhibition studies of purine biosynthesis were performed utilizing the method described by Henderson (16). The method is dependent on the observation that in the presence of azaserine, the only radioactive acid-soluble compounds formed from glycine-2-^{14}C are FGAR[2] and a very small amount of glycinamide ribonucleotide (17). Separation of FGAR-^{14}C from free glycine was achieved by means of column chromatography (17). The estimation of intracellular levels of 5-phosphoribosyl-1-pyrophosphate (PRPP) was based on its enzymic reaction with adenine-8-^{14}C to form radioactive adenylic acid (AMP). The method[4] recently described by Henderson and Khoo (18) was employed and is essentially as follows. Tumor cells, 200 mg wet weight, were incubated in 10 ml of calcium-free Krebs-Ringer phosphate medium, pH 7.4, in 25 ml Erlenmeyer flasks which were shaken at 37° in an air atmosphere. To extract PRPP from the tumor cells, 1.0-ml aliquots of medium containing cells were removed by pipette from incubation vessels and rapidly transferred to 12 ml centrifuge tubes standing in boiling H_2O. After 30 sec, the tubes were removed and placed in iced water. Denatured protein was sedimented by centrifugation, leaving a clear supernatant containing the PRPP. This PRPP was then reacted enzymically with an excess of adenine-8-^{14}C to form AMP. A crude extract of Ehrlich ascites carcinoma cells containing AMP pyrophosphorylase activity was used for the assay (4, 9). The AMP was isolated by paper chromatography and measured by its radioactivity (9, 18). Under the conditions used, PRPP was limiting for this reaction. The amount of radioactive AMP found was a direct measure of the amount of PRPP originally present.

RESULTS

Survival Studies

In previous studies with arabinosyladenine (6, 7) it has been shown that this analog was less effective against tumor

[4] We thank Dr. Henderson for sending us details of this method prior to publication.

lines which have high levels of adenosine deaminase. In a comparative study of the deamination of adenine nucleosides by various mouse tissues and tumors, the rate of deamination of XA has been shown to vary greatly from one tumor line to another (8). The effects of XA on the survival times of mice bearing several different ascites cell tumors are shown in Table 1.

TABLE 1

Effect of XA on the survival time of mice bearing ascites tumors

Therapy with XA was initiated 48 hours after implantation of tumor cells and was continued once daily for the indicated number of days at a dose level of 25 mg/kg. Each group consisted of 10 mice. Average survival and standard deviation are given. Numbers in parentheses are volume of cells/mouse present in mice sacrificed 7 days after tumor inoculation.

	Survival time (days)		
Tumor	Controls	Number of treatments	Treated
TA3	10.4 ± 1.4 (0.48)	4	19.6 ± 3.8 (0.03)
		6	24.2 ± 6.1
Ehrlich	15.0 ± 3.6	4	23.5 ± 5.5
Mecca	10.5 ± 0.6	4	10.6 ± 0.7
		6	10.6 ± 1.2

In mice bearing TA3 or Ehrlich ascites tumor cells there was a consistent lengthening of the life span of the mice. No significant prolongation of survival time was obtained with mice bearing Mecca lymphosarcoma ascites tumor cells. Four treatments with 25 mg/kg temporarily suppressed the growth of either TA3 or Ehrlich ascites tumor cells. This carcinostatic action of XA could be extended by further treatments. But with extended treatment a marked weight loss occurred, although there was no other evidence of toxicity. The failure of XA to prolong the survival time of mice bearing Mecca lymphosarcoma ascites tumor cells could be due to the comparatively rapid deamination of the drug by this tumor line.

To determine whether the inhibition of tumor growth by XA was associated with metabolic alterations in purine metabolism, the effect of XA on the *in vivo* incorporation of radioactive adenine and glycine into purine nucleotides of TA3 ascites tumor cells was measured. The labeled precursors were administered to the mice at indicated times following the injection of a single dose of 25 mg/kg of XA. The tumor cells were harvested after 60 min exposure to the isotope and the specific activity of nucleic acid adenine and guanine was determined as previously described (10). Treatment with XA markedly depressed the rate of incorporation of adenine-8-C^{14} into both RNA and DNA purines (Table 2). The rate of utilization of glycine for the formation of nucleic acid adenine and guanine was decreased by 50–90% without any effect on the incorporation of glycine into residual protein. The effect of a series of concentrations of XA on the incorporation of glycine and adenine into the acid-soluble fraction and nucleic acid adenine following incubation of TA3 ascites tumor cells *in vitro* was followed (Table 3). With increasing concentrations of XA, there was increased inhibition of the incorporation of either adenine or glycine into both the acid-soluble and the nucleic acid fractions. XA at 2 mM caused an inhibition of approximately 90%. The effect on the incorporation of glycine into protein was negligible at all concentrations of XA employed. It was reasoned that the metabolic effects observed with XA could result from a replacement of the adenine nucleotide pool of the cells by XA nucleotides. TA3 ascites

TABLE 2

Incorporation of adenine-8-^{14}C and glycine-2-^{14}C into nucleic acids of TA3 ascites cells treated with XA in vivo

Control mice were given an injection of 0.25 ml of saline whereas treated mice received 25 mg/kg of XA. At the indicated time intervals 75 μg (1.6 μC/μmole) of adenine-8-^{14}C or 200 μg glycine-2-^{14}C (1 μC/μmole) were administered to each mouse. Mice were killed 1 hour after injection of labeled precursor.

		Cpm/μmole $\times 10^{-2}$				
		RNA		DNA		
^{14}C Precursor	Treatment	Adenine	Guanine	Adenine	Guanine	Cpm/mg Protein
Adenine-8-^{14}C	Control	121	9.8	27	2.7	—
	XA, 1 hr	50	4.1	10.8	1.5	—
	XA, 3 hr	62	7.0	8.7	1.6	—
Glycine-2-^{14}C	Control	10.4	16.6	3.4	7.6	1.7×10^{-3}
	XA, 1 hr	2.5	3.9	1.1	1.6	2.1×10^{-3}
	XA, 3 hr	6.1	7.7	2.0	2.3	2.0×10^{-3}

TABLE 3

Effects of varied concentration of XA on the incorporation of glycine-2-^{14}C or adenine-8-^{14}C into purine nucleotides by TA3 ascites tumor cells in vitro

Each figure is an average of results from duplicate incubations.

	Glycine-2-^{14}C			Adenine-8-^{14}C		
	Cpm/μmole $\times 10^{-2}$				Cpm/μmole $\times 10^{-2}$	
Precursor: XA (mM)	Acid-soluble adenine	Acid-insoluble adenine	Cpm/mg protein	Total AS (counts $\times 10^{-3}$)	RNA adenine	DNA adenine
0	61	9.1	2350	110	11	5
0.5	20	4.0	2550	85	5	2
1.0	5.1	3.1	2400	41	2	1
2.0	3.6	0.9	2300	28	0.9	0.4

cells were incubated with various concentrations of XA for 60 min at 37°. Aliquots were taken at 30 and 60 min. After they had been washed and centrifuged, the cells were extracted twice with 5% TCA. The nucleotides were separated from inorganic phosphate by adsorption on charcoal (15). The TCA supernatant after centrifugation was decanted into a centrifuge tube containing 30 mg activated Norit, mixed, and centrifuged. To recover the labile phosphorous of the nucleotides (7 min acid-labile nucleotide phosphate) the charcoal was suspended in 1 N H_2SO_4 and the mixture was heated for 7 min at 100°. The mixture was cooled and centrifuged, and a 2-ml aliquot was assayed for its inorganic phosphate content. In the presence of increasing concentrations of XA there was a corresponding increase in the level of nucleotide phosphates (Table 4). The amount of labile nucleotide phosphate in the cells was increased by 80% in the presence of 2 mM XA. These results suggested that XA was phosphorylated within the cell and caused an increase in the total nucleotide phosphates. Chromatography, of the acid-soluble fraction of TA3 ascites cells incubated with 2 mM XA, on a Dowex-1-formate column indicated that the main increase in the acid-soluble nucleotides was in the ATP fraction. Periodate degradation

TABLE 4

The effects of XA on nucleotide phosphate levels

Ascites cell suspensions in 5 ml Robinson's medium containing 0.02 M $NaHCO_3$ and 0.1% glucose were incubated with or without xylosyladenine. The mixtures were placed in 25-ml Erlenmeyer flasks and incubated with shaking for 60 min at 37°. The nucleotides from the acid-soluble fractions were separated as described in the text.

Xylosyl adenine (mM)	10 mnp[a]
0	3.8
0.5	4.5
1.0	5.9
2.0	6.8

[a] 10 mnp refers to μmoles of 10-min acid-labile nucleoside phosphate per milliliter cells.

followed by chromatography was utilized to identify this nucleotide. The acid-soluble fraction of cells which had been incubated *in vitro* with 2 mM XA was treated with periodate and glycine buffer (19), to oxidize the riboside-5-phosphates, and further chromatographed on a Dowex-1-formate column (Fig. 1). It was demonstrated that this procedure would completely remove ribosyl derivatives. After elution of free bases and nucleosides (fraction 6–11) a major ultraviolet absorbing area (fractions 55–64), eluted in the region indicating a triphosphate, was obtained. This peak was completely absent when the cells were incubated without XA. Fractions 55–64 were pooled and adsorbed to acid-washed Norit; elution was carried out with ethanol–ammonia–H_2O (50–3–47). The eluate was lyophilized and the residue taken up in H_2O. The absorption spectra of this fraction were similar to that of adenosine triphosphate. (A250/260 was 0.82 and 280/260 was 0.17 at pH 2). Assuming a molar extinction coefficient of 14.5×10^3 at 260 mμ, phosphorus analysis showed that this compound contained 2.9 μmoles of total phosphate and 1.94 μmoles of acid labile phosphate per micromole of adenine. When the compound was subjected to paper chromatography with an isobutyric acid containing-solvent (20), it moved as a single UV absorbing spot with a R_{ATP} of 1.08 and a

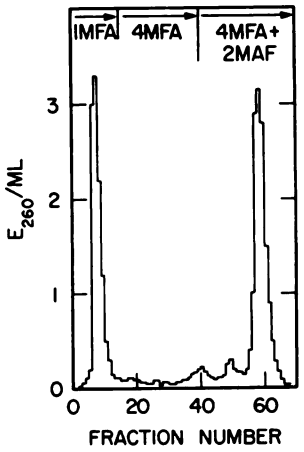

FIG. 1. *Ion exchange chromatogram of the soluble nucleotide fraction of TA3 ascites tumor cells incubated in vitro with XA*

Packed TA3 ascites cells (2.5 ml) were incubated in 20 ml of Robinson's medium containing 10 mg of XA for 60 min at 37°. The cells, collected by centrifugation, were extracted with 5 ml cold 2% $HClO_4$. The precipitate was washed with 3 ml of cold 2% $HClO_4$ and the combined supernatants were neutralized with KOH. The solution was chilled, $KClO_4$ was removed, and 150 μmoles of $NaIO_3$ was added. After incubation for 1 hr at room temperature, 100 μmoles of glucose was added to remove the excess periodate and finally the mixture was made alkaline with glycine buffer (19). The mixture was then applied on a Dowex-1-formate column (120 mm × 10 mm) and elution was carried out using the formic acid-ammonium formate gradient system. A 300-ml mixer was used, and 8-ml fractions were collected.

R_{dATP} of 0.93. On paper chromatograms developed with a borate-containing solvent (21) the compound behaved similar to 2′-deoxy-ATP. On the basis of these findings it is assumed that the compound is the triphosphate of XA. Under the conditions used for the incubation, approximately 2.6 μmoles of the triphosphate accumulated per gram wet weight of tumor cells. This is in contrast to the results of Klenow (22) and Shigeura and Gordon (23) with 3′-deoxyadenosine, where large amounts of the mono- and diphosphates were also obtained. However, their incubations with 3′-deoxyadenosine were continued for 3 hr under conditions where the glucose supply

would become exhausted; longer incubation with XA,[3] especially *in vivo* where the supply of glucose is limited, resulted in a decreased amount of triphosphate and a corresponding increase in the mono- and diphosphates. Further identification of the isolated compound as a high-energy triphosphate was provided by the observation of a rapid emission of light with crude firefly extracts comparable to that obtained with deoxy-ATP under the same conditions.

The utilization of preformed purines for nucleotide synthesis involves the participation of PRPP. Since XA inhibits the utilization of preformed purines for both RNA and DNA synthesis, it was possible that the drug might be inhibiting the synthesis of PRPP. An *in vitro* test system, in which the utilization of adenine-8-^{14}C for nucleotide formation was measured, was used as an indirect assay of the PRPP supply. TA3 ascites tumor cells were incubated *in vitro* with 1 mM XA. To both a control without XA and to the XA-treated cells, adenine-8-^{14}C was added at various times and incubated for a period of 10 min, the acid soluble nucleotides were isolated. The radioactivity in the nucleotide fraction following a 10-min pulse with adenine-8-^{14}C was determined. When adenine was added at the same time as the XA the radioactivity found in the nucleotide fraction was reduced to 40% of the control obtained in the absence of added XA (Table 5). When the cells were preincubated for 10 min or longer with XA before the addition of adenine-8-^{14}C, nucleotide formation was reduced to 20% of the control. With purified XATP prepared as above, we have demonstrated in cell-free extracts that this is the active substance which inhibited the formation of PRPP[3] (9).

TABLE 5

Incorporation of adenine-8-^{14}C into acid-soluble nucleotides of TA3 cells in vitro at different times after the addition of 1 mM XA

Time of addition of adenine-8-^{14}C after XA	% Control radioactivity in acid-soluble nucleotides
Control	100
0	39
10	22
15	20
20	21
30	21

In addition to their effect on PRPP synthesis, XA nucleotides might be acting as feedback inhibitors of purine biosynthesis. This activity can be tested by measuring the accumulation of glycine-^{14}C as formylglycinamide ribotide (FGAR), in azaserine-treated ascites cells. The effect of XA on the accumulation of FGAR was compared to that of adenosine, a known feedback inhibitor (16). At a concentration of 1 mM, XA was as effective an inhibitor of FGAR accumulation as was adenosine (Table 6). Either one produced a 70% reduction of the incorporation of glycine-^{14}C into FGAR.

TABLE 6

Feedback inhibition of purine synthesis by adenosine and xylosyl adenine

Tumor cells were incubated with 5×10^{-3} glucose, 10^{-3} M glutamine, 2×10^{-3} M glycine-^{14}C, and 6.4×10^{-6} M azaserine. Inhibitor concentration was 10^{-3} M.

Additions	Total cpm in FGAR	% Inhibition
Nil	25,000	0
Adenosine	7,200	71
XA	7,500	70

The observation that, in cell-free enzyme systems, purified XATP appeared to inhibit the formation of PRPP from ribose-5-phosphate[3] without having any effect on the condensation of PRPP with nucleotide precursors, led us to attempt to measure the intracellular levels of PRPP in cells treated with XA. We have employed the method of Henderson and Khoo (18) in which PRPP is extracted from cells, following *in vitro* incubation in the presence of glucose, and allowed to react with excess adenine-8-^{14}C to form radioactive AMP. The amount of radioactivity in the AMP is a measure of the amount of PRPP originally present in the cells. Henderson and Khoo (18) found that, in the absence of

added glucose, the level of PRPP in ascites cells is almost negligible. Addition of glucose caused an increase in the amount of PRPP present in the cell. This level reached a plateau (2–3 μmoles/g wet weight) 30 min after the addition of glucose and did not change substantially for more than 30 min thereafter. Under the conditions used in the present experiments, the plateau level of PRPP was not reached until 60 min after addition of glucose and was only about half of that reported by Henderson and Khoo (18). Table 7 shows

TABLE 7

PRPP levels in Ehrlich ascites tumor cells incubated in vitro with XA

Tumor cells were incubated in Krebs-Ringer phosphate medium, pH 7.4, for 30 min in an air atmosphere. Isolation and determination of PRPP were as described in the text.

Conditions	PRPP (μmoles/g)
No Glucose	0.012
Glucose, 10 mM	0.770
Glucose + 1 mM XA	0.058
Glucose + 2 mM XA	0.010

the levels of PRPP found in cells incubated *in vitro* for 30 min in the presence of both glucose and XA. Addition of XA at the same time as the glucose completely inhibited the production of PRPP. This experiment demonstrated that XA prevented the formation of PRPP but did not show what effect the addition of XA might exhibit on a significant level of PRPP already present in the cell. The results of such an experiment are given in Fig. 2. Ehrlich ascites tumor cells were incubated in Krebs-Ringer phosphate containing 10 mM glucose. One-milliliter samples were taken every 10 min for estimation of the amount of PRPP present in the cells. At 30 min the cell suspension was divided into two aliquots. To one aliquot 2 mM XA was added, while the other was used as a control. Samples were taken at intervals from each flask until the level of PRPP in the control sample reached a plateau. From

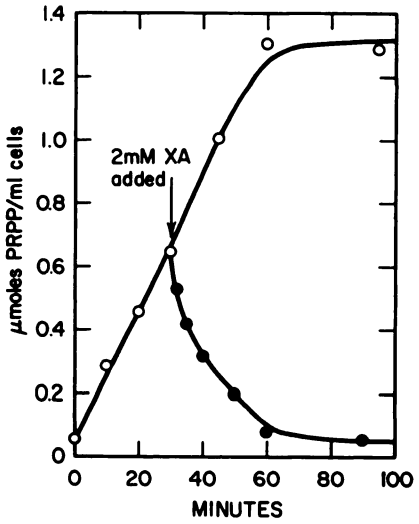

FIG. 2. *PRPP synthesis in Ehrlich ascites tumor cells in vitro.*

Tumor cells, 200 mg wet weight, were incubated at 37° in an air atmosphere in 10 ml of Calcium-free Krebs-Ringer phosphate medium, pH 7.4 with 10 mM glucose present; 2 mM XA was added at the indicated time. ○——○ Control; ●——● 2 mM XA present.

the results presented in Fig. 2 it is apparent that addition of XA stopped further synthesis of PRPP and caused a rapid depletion of any PRPP already present in the cells. By 30 min after the addition of XA, the level of PRPP found in the cells was at the low level found in the absence of glucose.

DISCUSSION

As in the case of some other purine antimetabolites, it appears that XA has its metabolic effects after conversion to nucleotide form. *In vitro,* with an adequate glucose supply, the analog was all converted to XATP and little or no XADP or XAMP was found. No conversion to free base occurred (8). This kinase activity was much more rapid than was found with either arabinosyl adenine or lyxosyl adenine.[5] Conversion to XATP was less complete *in vivo,* where glucose supply might be expected to be somewhat limiting. XATP was found to be a good inhibitor of

[5] J. L. York and G. A. LePage, unpublished.

both purine synthesis (feedback) and PRPP synthesis. The feedback inhibition can probably be discounted as unimportant, since adenosine cannot produce the increased survival times observed in mice treated with XA. The effects on PRPP supply appear more likely to be the primary reason for tumor inhibition, since this would limit availability of purine nucleotides synthesized either *de novo* or from preformed purines. Rapid host weight loss at high doses of XA still suggests effects in the area of protein synthesis. These may be secondary to the effects on nucleotides and polynucleotides.

In arabinosyl adenine the "fraudulent" structure results from transposition of the hydroxyl group on carbon 2 of the sugar. In xylosyl adenine the transposition is at carbon 3 of the sugar. It is evident that these relatively subtle structural changes have produced analogs with quite different metabolic effects.

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