Effects of 6-Azaauridine on Nucleotides, Orotic Acid, and Orotidine in L5178Y Mouse Lymphoma Cells in Vitro

Claude M. Janeway and Sungman Cha

Division of Biology and Medicine, Brown University, Providence, Rhode Island 02912

SUMMARY

Murine lymphoma L5178Y cells in culture were exposed to 5 μM 6-azaauridine (6-aza-UR) for up to 8 hr. The nucleotides in the acid-soluble extracts were determined by high-pressure liquid chromatography. The concentrations of uracil and cytosine nucleotides decreased rapidly in the treated cells. Adenosine 5′-triphosphate and adenosine 5′-diphosphate increased steadily, whereas guanosine 5′-diphosphate decreased for a few hr before returning to the normal level. Orotic acid and orotidine, but no detectable amount of orotidine monophosphate (OMP), accumulated in treated cells. In homogenates of the cells harvested from ascitic fluids of mice, the presence of a membrane-bound phosphatase with activity for OMP was demonstrated, which may be responsible for the lack of OMP accumulation. When incubated with the homogenate, [7-14C]OMP was converted to [7-14C]orotidine in the absence of inorganic phosphate, but it was converted to [7-14C]orotic acid in the presence of phosphate, suggesting the occurrence of an orotidine phosphorlyase. The increase in adenosine 5′-triphosphate levels and the rapid decrease in the pyrimidine nucleotides observed after treatment with 6-aza-UR may be responsible for the observation (Brenckman et al., Biochem. Biophys. Res. Commun., 52: 1368-1373, 1973) that 6-aza-UR potentiates the activity of 1-β-D-arabinofuranosylcytosine only when 6-aza-UR is added prior to 1-β-D-arabinofuranosylcytosine.

INTRODUCTION

Various aspects of the clinically useful pyrimidine antimetabolite, 6-aza-UR, have been extensively reviewed by several investigators (16, 19, 28). Taking advantage of the recent improvements (e.g., dual wavelength monitoring) in high-pressure liquid chromatography, we have undertaken a study of the effects of 6-aza-UR on the nucleotide pools of mouse lymphoma L5178Y cells in culture. The simplicity and versatility of this chromatographic technique allowed us to determine quantitatively many nucleotides and to follow the time course of changes in metabolic concentrations, particularly those of orotic acid, orotidine, and OMP.

In addition to drastic changes in pyrimidine nucleotides and their precursors, significant effects have been observed in the purine nucleotide concentrations. A working hypothesis is offered to explain the mechanism of accumulation of orotic acid and orotidine. Plausible explanations are also offered for previously observed but unexplained findings, i.e., the potentiating of the effects of ara-C by 6-aza-UR when 6-aza-UR is given prior to ara-C (2).

MATERIALS AND METHODS

Chemicals. Fischer’s medium, horse serum, and antibiotics necessary for the culture of L5178Y cells were obtained from Grand Island Biological Co., Grand Island, N. Y. 6-Aza-UR, 6-aza-UMP, orotidine, and OMP were purchased from Calbiochem, Los Angeles, Calif. [7-14C]OMP (39.3 mCi/mmol) was obtained from New England Nuclear, Boston, Mass. OMP pyrophosphorylase (orotate phosphoribosyltransferase, EC 2.4.2.10) from baker’s yeast and 5-fluoroorotate were purchased from P-L Biochemicals, Inc., Milwaukee, Wis. The enzyme was purified further, approximately 10-fold, by the use of a Sephadex G-200 column eluted with a 0.2 M Tris-Cl buffer containing 0.05 M MgCl2 at pH 8.0. Even after purification, the preparation had considerable OMP decarboxylase (EC 4.1.1.23) activity. The enzyme was assayed spectrophotometrically by the method of Dahl et al. (8) with 5-fluoroorotate as substrate.

All other nucleotide standards used for identification of peaks on high-pressure liquid chromatography were purchased from Sigma Chemical Co., St. Louis, Mo.

Cell Culture and Extraction Conditions. The L5178Y cells were obtained from Dr. M-Y. Chu of Brown University. The cells were grown in culture in 500-ml bottles at 37° in Fischer’s medium supplemented with 10% horse serum, as described by Fischer and Sartorelli (12). Cell numbers were counted with a Model B Coulter counter (Coulter Electronics, Inc., Hialeah, Fla.). Exponentially growing cultures of L5178Y cells were exposed to 6-aza-UR (final concentration, 5 μM) for varying periods of time. Cells were collected by centrifugation and washed twice with cold 0.9% NaCl solution. The packed cells (approximately 5 × 107 cells) were extracted twice with 0.5 ml of trichloroacetic acid at 4°. The extracts were combined, and trichloroacetic acid was removed by extracting 3 times with water-saturated diethyl ether. The aqueous phase (pH 6.0) was lyophilized, was removed by extracting 3 times with water-saturated diethyl ether. The aqueous phase (pH 6.0) was lyophilized, and the solids were redissolved in 100 μl of water and kept frozen until analysis by high-pressure liquid chromatography.
Chromatographic Procedures for Nucleotides. Nucleotide levels in the acid-soluble extracts were determined by high-pressure liquid chromatography with a Varian Aeralograph LCS-1000 instrument equipped with a Partisil PX5 SAX column (Whatman, Inc., Clifton, N. J.). The nucleotides (usually 30 to 50 µl of the 100-µl extract) were eluted from the column with a potassium phosphate buffer (phosphoric acid adjusted to pH 4.5 with KOH) under the following conditions: low concentrate was 2 mM and high concentrate was 0.5 M; starting volume in the mixing chamber was 25 ml (low concentrate). The elution scheme used in these studies consisted of elution with low-concentrate buffer (2 mM) for 30 min at a flow rate of 25 ml/hr through the column (pump settings were 25 and 0 ml/hr for the column and gradient pumps, respectively) followed by a convex gradient from 2 mM to 0.5 M buffer at a flow rate of 40 ml/hr through the column (both column and gradient pump flow rates were 40 ml/hr). Elutions were carried out at a constant pH value of 4.5. During the gradient delay, the volume of buffer in the mixing chamber decreased from 25 to 12.5 ml and was maintained at this latter value throughout the remainder of the elution. UV absorption of the eluate was monitored at 2 wavelengths, 254 and 290 nm. The A254/A290 absorption ratios greatly facilitated identification of the peaks. At pH 4.5, the cytosine nucleotides and orotic acid absorb strongly at 290 nm. The extinction coefficient of orotic acid (8800) at 290 nm and pH 4.5 is much greater than those of orotidine (1900) and OMP (2300). Some nucleotide peaks were identified by comparison of retention time and by superimposition with known standards. The nucleotide concentrations were determined by comparison of the peak areas with those of a series of standard nucleotides by the use of a digitized planimeter (Model EGC, Numonics Corp., North Wales, Pa).

Identification of Orotic Acid by an Enzyme Peak Shift. An acid extract of cells treated with 6-aza-UR, containing 17 nmol of orotic acid and 11 nmol of orotidine, was incubated at room temperature for 5 hr with 0.01 unit of OMP pyrophosphorylase, 25 µmol of Tris-Cl (pH 7.9), and 10 µmole of MgCl₂, in the presence of 0.1 µmole of PRPP and 10 nmole of 6-aza-UMP in 0.5 ml. The proteins were precipitated by the addition of 0.05 ml of 50% perchloric acid, and the extract was neutralized with 5 N KOH. The extract (50 µl) was subjected to high-pressure liquid chromatography.

Metabolic Studies of OMP. L5178Y cells were also grown by the i.p. transplantation of 1000 tumor cells in C3H/J × DBA/2J F₁ mice (The Jackson Laboratory, Bar Harbor, Maine). Cells were harvested 15 days after inoculation. The cells were suspended in cold 0.9% NaCl solution and centrifuged. Contaminating erythrocytes were removed by exposing the cells to a hypotonic solution for 20 sec, as described by Scholar and Calabresi (27). After being washed twice with cold 0.9% NaCl solution an aliquot of the cells was homogenized in 0.1 M Tris-Cl buffer (pH 7.4), and the remainder was homogenized in 0.05 M potassium phosphate buffer (pH 7.4). Both homogenates were dialyzed against their respective buffers overnight. The dialyzed homogenates, low-speed supernatant (800 × g, 5 min), and high-speed supernatant (100,000 × g, 5 hr) were used.

RESULTS

The effects of 6-aza-UR on the growth of L5178Y cells in Fischer's medium are shown in Chart 1. On the basis of these results, 5 µM was chosen as the concentration of the drug for studies of its effects on various nucleotides. A high-pressure liquid chromatography profile of an acid-soluble extract of cells in exponential growth phase is shown in Chart 2, top. All but a few peaks have been identified by the methods described previously. Contrary to results from other laboratories (4, 22, 30), UDP eluted before CDP, and UTP eluted before CTP, while UMP eluted after CPM. These discrepancies may be due to the different characteristics of the different column materials used, as well as the poor buffering capacity of the low-concentrate buffer. Also, unlike the method used in many other laboratories, no pH gradient was used in this work. The large peak emerging immediately after AMP has a retention time...
identical with that of IMP, but it has not been positively identified at this time. Concentrations of major nucleotides estimated from this and similar chromatograms are presented in Table 1.

Shown in Chart 2, bottom, is the nucleotide profile of cells grown for 2 hr in the presence of 5 μM 6-aza-UR. The most obvious differences between this profile and that of the control cells are the marked decreases in the sizes of all pyrimidine nucleotide peaks (UTP, UDP, CTP, CDP, and UDP-glucose), increases in ATP and ADP peaks, and the appearance of 2 new peaks in the nucleoside monophosphate region. Nelson (22) also observed similar peaks and tentatively assigned the larger of the 2 peaks to orotidine. These 2 new peaks have strong absorption at 290 nm and pH 4.5. The first peak was identified as orotidine by superimposition of an authentic sample. The second of the 2, with a much higher A_{290}/A_{254} ratio, was identified as orotic acid by the method to be described below.

For positive identification of the orotic acid peak, an enzymic peak shift technique was applied, as described in "Materials and Methods", and the results are shown in Chart 3. Under the experimental conditions, decarboxylation of OMP to UMP was largely blocked by 6-aza-UMP. Upon this enzymic treatment, the major 290-nm absorbing peak in the control extract (marked as orotic acid in Chart 3, top) disappeared, and a new peak with a retention time identical with that of OMP appeared, (Chart 3, bottom). The 290-nm absorbing peak, which eluted before the orotic

---

### Table 1

| Nucleotide concentration of L5178Y cells |
|-------------------------------|------------------|
| Nucleotide                  | μmoles/1.5×10^6 cells |
| ATP                          | 1.06 ± 0.46       |
| ADP                          | 0.14 ± 0.09       |
| GTP                          | 0.29 ± 0.11       |
| GDP                          | 0.02 ± 0.02       |
| UTP                          | 0.44 ± 0.20       |
| UDP                          | 0.06 ± 0.04       |
| CTP                          | 0.21 ± 0.06       |
| CDP                          | 0.38 ± 0.06       |
| UDP-glucose                  | 0.15 ± 0.10       |

* Average of 8 experiments. One ml of packed cells equals approximately 1.5×10^6 cells.

* Mean ± S.D.

* A maximum value, since other minor peaks, e.g., xanthosine monophosphate, are usually poorly resolved.
6-aza-UR Effects in L5178Y

Acid peak, remained unchanged, while the peak of added 6-aza-UMP appeared. These findings confirm the identities of the orotic acid and orotidine peaks shown in Chart 1.

Chart 4 summarizes the time sequence of changes in the concentrations of various nucleotides after addition of 6-aza-UR. The pyrimidine nucleotides decreased markedly, as expected. On the other hand, the ATP concentration increased steadily, while the GTP concentration decreased for a few hr and then returned to the normal level. Orotic acid and orotidine, which are undetectable in the control by these methods, also accumulated. Orotic acid tends to accumulate before orotidine, but eventually orotidine exceeds orotic acid.

Since 6-aza-UR is believed to exert its action after conversion to 6-aza-UMP by blocking decarboxylation of OMP, one might expect to see OMP accumulation; however, OMP does not accumulate in detectable amounts. As will be discussed later, this suggests the presence of an active phosphatase, possibly 1 of the 5'-nucleoside monophosphatases, possessing a low $K_m$ for OMP.

To find explanations for the accumulation of orotidine and orotic acid but not of OMP, we carried out the following experiments. As may be seen in Table 2, [7-14C]OMP was completely metabolized during a 2-hr incubation at 37° with an aliquot of the low-speed supernatant of the homogenate dialyzed in Tris buffer. In the absence of 6-aza-UMP, OMP was almost completely decarboxylated to UMP, and the radioactivity was lost as CO$_2$. Only a small amount (7%) of the radioactivity was present as orotidine. In the presence of 0.01 mM 6-aza-UMP, however, 65% of the original radioactivity was converted to orotidine, but no significant amount was converted to orotic acid. It appears that 6-aza-UMP did not block the decarboxylation completely, and the radioactivity that was not accounted for as orotidine plus OMP was lost as CO$_2$. Similar experiments were also carried out in the presence of 6-aza-UMP for various periods of time, and the results are shown in Chart 5, left. In a similar experiment, in which the phosphate buffer was
used throughout the homogenization, dialysis, and incubation, more orotic acid was formed than orotidine (Chart 5, right) suggesting that orotidine was converted to orotic acid by a phosphorylase. Chart 6 shows the results of experiments in which Tris-Cl buffer was used throughout the procedure. The putative phosphatase present in the homogenate (as indicated in Chart 6, left) was removed by centrifugation at a high speed (100,000 \( \times g \), 5 hr) (Chart 6, right).

**DISCUSSION**

6-Aza-UR exerts its effects after phosphorylation to 6-aza-UMP, which in turn inhibits OMP decarboxylation, thus preventing the de novo biosynthesis of pyrimidine nucleotides (14, 15, 24). The drastic decrease in the concentrations of the pyrimidine nucleotides observed above in 6-aza-UR-treated cells are, therefore, consistent with these previous observations. Nevertheless, the depletion of the pyrimidine nucleotides is very rapid and is almost complete in less than 1 hr.

Since 6-aza-UMP inhibits OMP decarboxylase (14, 15, 24), it is natural to expect an accumulation of OMP. As discussed previously, however, no detectable amounts of OMP accumulated at any time after 6-aza-UR treatment. Instead, orotic acid and orotidine accumulated. Accumulation of orotic acid and orotidine has been observed after 6-aza-UR treatment in bacterial culture media (29), experimental tumors of mice (24), and human urine (5, 11). The simplest explanation for the accumulation of these compounds, but not of OMP, may be that they are the degradation products of OMP that accumulate after 6-aza-UR treatment. The accumulation of OMP was demonstrated in the particle-free supernatant fraction of L5178Y cells (24) and of rat liver (1). However, to the best of our knowledge, detectable accumulation of OMP has never been demonstrated in any intact cell. Therefore, it seems appropriate to raise questions as to how orotic acid and orotidine are formed.

The results presented in Table 2 and Charts 5 and 6 demonstrate that orotidine can be formed rapidly from OMP in homogenates of L5178Y cells and suggest the existence of a membrane-bound phosphatase. This is consistent with the observations of other investigators cited previously.

For the mechanism of accumulation of orotidine and orotic acid in the 6-aza-UR-treated cells, we propose the following working hypothesis on the basis of available evidence, as illustrated in Chart 7.

First, de novo synthesis of orotic acid increases as the consequence of a series of events. 6-aza-UMP, the active metabolite of 6-aza-UR, inhibits OMP decarboxylation. UTP and CTP concentrations decrease. The first and rate-limiting step of pyrimidine biosynthesis, the aspartate carbamyltransferase section, is stimulated by the removal of feedback inhibition by UTP and CTP (3), as well as by the possible increase in the amount of the enzyme, as shown in Sarcoma 180 cells by Ennis and Lubin (10).

Second, the OMP pyrophosphorylase reaction may be inhibited as a result of the inhibition of OMP decarboxylase by 6-aza-UMP. There is evidence that these 2 enzymes normally exist as a complex (26) and that OMP formed from orotic acid by the pyrophosphorylase reaction is directly channeled to the carboxylase without a rapid exchange with exogenous free OMP in the medium (31). If this is so, upon inhibition of the decarboxylase the endog-
enous OMP presumably bound to the enzyme complex would increase to a saturating level before the concentration of the free OMP in the medium increases significantly. Then, this enzyme-bound OMP might serve as a product inhibitor of the pyrophosphorylase. At the same time, 6-aza-UMP might also serve as an alternative product inhibitor. In addition to this product inhibition, it is conceivable that the inhibition of the decarboxylase moiety of the enzyme complex might cause simultaneous inhibition of pyrophosphorylase by a conformational change. The validity of these speculations, however, must be tested by future experimentation. The pyrophosphatase reaction is readily reversible with \( K_{\text{m}} \) of 0.12 in the direction of OMP formation (13). Nevertheless, the notion that orotic acid may accumulate simply due to the increased OMP concentration and by the reversal of the pyrophosphatase reaction appears to be untenable, because there is no evidence that OMP actually accumulates to a significant degree in treated cells and because the concentration of PP\(_i\) is very low in most cells.

Third, free OMP is rapidly converted to orotidine. As the endogenous OMP reaches a saturating level, it will dissociate from the enzyme complex and then be rapidly dephosphorylated to orotidine by a membrane-bound phosphatase. Thus, the concentration of free OMP is kept low.

Finally, orotidine and orotate are interconverted by a phosphorylase. This putative phosphorylase may or may not be the same enzyme as the uridine phosphorylase described by Pontis et al. (25).

The changes in the concentrations of the purine nucleotides were unexpected findings. ATP and ADP increased steadily for at least 8 hr, while guanine nucleotides (GTP and GDP) decreased for a few hr before returning to control levels. These findings are superficially analogous to those of Nelson and Parks (23), which showed that UTP increased in Sarcoma 180 cells after treatment with 6-methylmercaptopurine ribonucleoside. Nelson and Parks attributed this to the increased availability of PRPP for pyrimidine biosynthesis due to the inhibition of purine nucleotide biosynthesis by the 5'-monophosphate derivative of 6-methylmercaptopurine ribonucleoside. However, in this situation one would expect to find a decrease of PRPP rather than an increase, because the increased concentration of orotic acid may cause increased consumption of PRPP by the OMP pyrophosphorylase reaction. Furthermore, if the increases in adenine nucleotides were due to the stimulation of purine biosynthesis by an increase in PRPP, one would expect a concurrent increase in GTP, which is contrary to the previous observation. Indeed, our preliminary experiments to determine PRPP concentrations by the method of Henderson and Khoo (17) in the extracts prepared by the method of Hisata (18) revealed no indication of an increase in the concentrations (approximately 75 \( \mu M \)) of PRPP after 6-aza-UR treatment. Since the above data are expressed on a per cell basis, it is conceivable that the ATP increase merely reflects increases in cell size following 6-aza-UR treatment, whereas the ATP concentration per se remained constant. While the answer to this question must await cell size distribution studies, the slower increase in ADP and the decrease in GTP do not support this explanation. A more plausible explanation for the increase of ATP is that the consumption of ATP may be spared due to the lack of pyrimidine nucleotide metabolism beyond UMP and the consequential cessation of nucleic acid synthesis. The fact that ATP increases relatively more than ADP is also consistent with this possibility of an ATP-sparing effect.

Regardless of the mechanism of the ATP increase, this phenomenon, together with the decrease in pyrimidine nucleotides, may explain an interesting observation made by Brenckman et al. (2). These investigators reported that 6-aza-UR and ara-C exhibited a potentiation when L5178Y cells were exposed to 6-aza-UR before ara-C, an additive effect if ara-C preceded 6-aza-UR, and a less-than-additive effect if the 2 drugs were administered simultaneously. It is generally accepted that ara-C is converted to ara-CMP by deoxycytidine kinase and then to ara-CTP by various nucleotide kinases (6, 7). Therefore, depletions of deoxycytidine compounds as a consequence of the decrease in CTP may result in stimulation of ara-CTP formation by decreasing the levels of substrates that compete for various enzymes in the pathway. Since the conversion of ara-C to ara-CTP requires ATP, increases in ATP may also facilitate this conversion. The \( K_{\text{m}} \) value of ATP for ara-CMP formation by calf thymus deoxycytidine kinase was reported to be 2 \( \times \) 10\(^{-4} \) M (9, 21), and that for dCDP formation by rabbit muscle deoxycytidylylase kinase was reported to be 3.1 \( \times \) 10\(^{-4} \) M (20). Although the \( K_{\text{m}} \) values of these enzymes from L5178Y cells for ara-C or its derivatives as a second substrate are not known, the ATP concentration of 1 \( \mu M \) in the control cells is 5-fold greater than the \( K_{\text{m}} \) value of ATP for the calf thymus deoxycytidine kinase cited above. Therefore, a 2-fold increase in the ATP concentration could significantly influence the rate of conversion of ara-C to ara-CMP.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Dr. M.-Y. Chu for her invaluable contributions throughout these studies, Dr. Woo Ik Hwang for carrying out the initial phase of this work, Dr. G. A. Fischer and Dr. G. W. Crabtree for valuable advice, and Arpie Shiragian for her assistance in the high-pressure liquid chromatography.

REFERENCES

C. M. Janeway and S. Cha


Effects of 6-Azauridine on Nucleotides, Orotic Acid, and Orotidine in L5178Y Mouse Lymphoma Cells  \textit{in Vitro}

Claude M. Janeway and Sungman Cha


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/37/12/4382

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.