Alterations in Human Pyrimidine Metabolism as a Result of Therapy with 6-Azauridine*

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SUMMARY

Biochemical procedures are described which have been found useful in the study of normal pyrimidine metabolism as influenced by 6-azauridine. One technic involves the trapping of radioactive carbon dioxide liberated from orotic acid-7-C\textsuperscript{14} during the conversion of this compound to uridine nucleotides by isolated leukocytes in vitro. The susceptibility of this conversion to inhibition by azauridine 5'-phosphate derived metabolically from azauridine in vivo or in vitro has been measured.

The accumulation of orotic acid and orotidine by patients with leukemia treated with azauridine has been quantitated by ion-exchange analysis of the urine from these patients. Another indication of the effect of azauridine on the metabolism of orotic acid has been obtained by intravenous injection of a tracer dose of orotic acid-7-C\textsuperscript{14}. Pre-treatment with azauridine of patients with neoplastic disease decreased the conversion of this pyrimidine to respiratory CO\textsubscript{2} and uridine nucleotides and increased the urinary excretion of unchanged orotic acid.

The antineoplastic effects of 6-azauridine (AzUR) have been demonstrated in a number of experimental murine neoplasms (7, 12). This pyrimidine analog, after metabolic conversion to azauridine 5'-phosphate, azauridytic acid (AzUMP), is believed to exert its effect on tumor growth primarily by inhibition of the enzyme orotidylic acid decarboxylase (5, 9) (Chart 1). This enzyme forms uridine 5'-phosphate (UMP) from the ribonucleotide of orotic acid, orotidine 5'-phosphate (OMP). In preliminary clinical studies, azauridine has produced evidence of temporary objective improvement in a number of patients with leukemia (13). Concurrent with these clinical trials, in this laboratory (1, 4) and elsewhere (2, 10), a number of biochemical tests were performed on patients to determine whether a similar biochemical blockade is produced in human subjects, as had been observed in experimental animals. In addition, it was of importance to determine the nature of the effect of the antimetabolite on certain normal cells and on the body as a whole, as well as on neoplastic cells. Such information could have relevance to the selection of patients for therapy and could serve as a guide in choosing between different modalities of treatment with AzUR.

Three approaches have been used. The first method tests the rate of the metabolic conversion of orotic acid-\textsuperscript{14}CO\textsubscript{2} to uridine nucleotides and \textsuperscript{14}CO\textsubscript{2} by whole cell suspensions \textit{in vitro} and the effect of either prior treatment of the patient with azauridine or the addition of the drug \textit{in vitro} on this metabolic activity. The second technic involves the quantitation of the urinary excretion of

[Diagram of pyrimidine metabolic pathways]
orotic acid and orotidine by patients and the correlation of these values with the dosage of azauridine. The third test provides a measure of the effect of this analog on the manner in which the body disposes of an intravenous injection of orotic acid-C\textsubscript{14}0O\textsubscript{H}. In this test, the excretion of radioactivity as C\textsuperscript{14}O\textsubscript{2} via the lungs and as unchanged orotic acid via the kidneys is compared.

Certain portions of this work have been presented in preliminary form elsewhere (1, 4).

MATERIALS AND METHODS

Orotic acid-C\textsubscript{14}00H (orotic acid-7-C\textsuperscript{14}; 6.8 \(\mu\text{c} / \mu\text{mole}) was purchased from the New England Nuclear Corporation. Azauridine was either donated by E. R. Squibb and Sons or provided through the Cancer Chemotherapy National Service Center of the Public Health Service (Bethesda, Maryland) from the California Corporation for Biochemical Research. Radioactivity determinations were made on aliquots of aqueous solutions in 10 ml. of a mixture of toluene and absolute ethanol (2:1) containing, per liter, 2.7 gm. of 2,5-diphenyloxazole (DPO) and 33 mg. of 1,4-di-(2-[5-phenyloxazole])-benzene (POPOP). A Liquid Phosphor Counter (Technical Measurements Company, Inc., New Haven) was used for all radioactivity determinations.

Orotic acid metabolism in isolated cells.—Normal or leukemic leukocytes from peripheral blood were prepared by adding 1 volume of a solution containing dextran, 6 per cent, and sodium chloride, 0.85 per cent, to 4 volumes of heparinized whole blood. After standing at room temperature for 45-60 minutes in a tube placed at a 45° angle, the supernatant fluid containing the leukocytes was removed and centrifuged; the leukocytes were washed with incubation medium. Blood specimens of approximately 30 ml. were required from patients with leukocyte counts between 5,000 and 10,000 per cu. mm. of blood.

All reactions were carried out in Warburg flasks (single arm) of 15-ml capacity and with 17/20 joints; the gas phase was air. The center-well contained 0.2 ml. of sodium hydroxide solution (2 M) and the side-arm contained 0.3 ml. of perchloric acid solution (6 M). To the flasks, which were kept on ice, was added 2 ml. of a suspension of approximately 3 \(\times\) 10\textsuperscript{7} leucocytes or 2 \(\times\) 10\textsuperscript{5} erythrocytes in Krebs III buffer. Susceptibility of the cells to inhibition by azauridine was measured by adding 0.005 or 0.05 \(\mu\text{mole} of AzUR per ml. of reaction mixture. After incubation of all flasks for 10 min. in a Dubnoff metabolic shaker at 37° C., 0.1 \(\mu\text{mole} of orotic acid-7-C\textsuperscript{14} (100,000 counts/min) was added, and the incubation was continued for 60 min. The perchloric acid solution was then tipped in, and the flasks were shaken for an additional 10 min. to insure the release and absorption of any remaining carbon dioxide. Aliquots (0.1 ml.) of the sodium hydroxide solution of the center-well and of the incubation mixture were assayed for radioactivity in the manner described.

Assays for orotic acid and orotidine in urine.—Aliquots (1 ml.) of 24-hour urine specimens obtained from patients receiving AzUR were diluted with 9 ml. ammonium hydroxide solution (0.05 N) and passed through 5-ml. columns of Dowex-1 \(\times\) 4 (formate form). The columns were washed with 10 ml. of water and eluted, successively, with three 10-ml. portions of formic acid solutions (0.05 N and 0.1 N, respectively) and ten 10-ml. volumes of ammonium formate (0.3 M, pH 5), as indicated in Chart 2. The optical density of the eluates was read at 260 and at 280 m\(\mu\) in a Beckman DU spectrophotometer. The total output of these compounds in the urine during 24-hr. periods was calculated from values obtained with a control urine to which orotic acid and orotidine standards had been added. The concurrent administration of large amounts of UV-absorbing drugs or purines and pyrimidines in the diet was avoided.

Metabolic clearance of orotic acid-C\textsuperscript{14}00H.—The metabolism of injected orotic acid by patients with neoplastic disease was tested as follows. A solution of 10 \(\mu\text{mole} of orotic acid-7-C\textsuperscript{14} (5 \mu\text{c.}) in 5 ml. of water was injected intravenously, and collec-

1 The authors are grateful to Dr. L. H. Smith, Jr., for suggesting this experiment and for communicating preliminary results obtained with rats.

2 Pilot Chemical Corp., Waltham, Mass.
tions of respiratory carbon dioxide were made at intervals of 5 or 10 minutes for at least 1 hour by having the patient exhale through a saturated solution of barium hydroxide. Urine specimens were collected at hourly intervals for the first 3 hours (0–3 hr.), followed by a 3-hour collection (3–6 hr.) and an 18-hour collection (6–24 hr.). Urinary radioactivity determinations were corrected for quenching errors by internal standards. The effect of AzUR on the excretion pattern was determined by giving the patient intravenous injections of a 20 per cent solution of AzUR at a dosage level of 60 mg/kg, ½ hour before injecting the orotic acid. The precipitated barium carbonate from collection of the respiratory carbon dioxide was washed, dried, and, after being ground to a fine powder, 100 mg. was suspended in 20 ml. of a solution composed of toluene, 4 per cent DPO, and 0.05 per cent POPOP and 1 gm. of Cab-O-Sil as a thickening agent. Correction of the counting efficiency to that of aqueous samples was made with a calibrated barium carbonate standard.

RESULTS AND DISCUSSION

Before considering the results obtained with peripheral leukocytes from patients, it must be emphasized that the data reflect a summation of events in the conversion of orotic acid to uridine nucleotides and carbon dioxide. These values are not only influenced by differences in (a) the condensation of pyrophosphoryl ribose 5'-phosphate (PRPP) with orotic acid and (b) the subsequent decarboxylation of orotidylic acid, but also by differences in (c) the enzyme-producing system, in (d) the production of the PRPP, and (e) in the transport of the substrate, orotic acid, into intact cells. Likewise, the inhibition of the decarboxylation reaction by AzUR is dependent upon its accessibility to the intracellular phosphokinase which phosphorylates it and upon the activity of this enzyme; in addition, the kinase reaction is ATP-dependent, and, accordingly, maintenance of adequate levels of this nucleotide by the cells is essential to inhibition. Although certain disadvantages are created by these many variables which influence the outcome of the test, the method provides, nevertheless, an over-all measurement of the capabilities of the tissue and of the susceptibility of the key reaction to inhibition.

This method has been applied to the determination of the enzymatic activities of solid tumors and normal tissues; unfortunately, the heterogeneity of normal and neoplastic tissues casts serious doubts upon the interpretations of these results. Studies with peripheral blood cells, which lend themselves more readily to discrete separation, are more promising. However, it should be emphasized that the leukocyte suspensions are inherently heterogeneous and the method used does not give complete separation from platelets. Both of these limitations are of less consequence in the studies with acute leukemia, since preponderance of one cell type and thrombocytopenia were the rule.

For the specific measurement of orotidylic acid decarboxylase, a particle-free supernatant fraction of cell homogenates could be prepared and the enzymatic activity ascertained spectrophotometrically or by release of C14O2 with orotidylic acid-C14OOH used as a substrate (3). With such a frac-

![Chart 3](chart3.png)

**Chart 3.**—The relationship of cell concentration to the metabolism of orotic acid-C14OOH to C14O2 and uridine nucleotides. The preparation of the cell suspensions and the incubation conditions are described under "Methods." RBC = erythrocytes; CLL = leukocytes obtained from cases of chronic lymphocytic leukemia; CGL = leukocytes obtained from cases of chronic granulocytic leukemia.

*Cardoso et al.—Pyrimidine Metabolism and 6-Azauridine* 1553

4 Cab-O-Sil M-5, Godfrey L. Cabot, Inc., 77 Franklin St., Boston 10, Mass.
of this metabolic pathway (Charts 4 and 5). Chart 5 presents examples of the three classes of response obtained with peripheral leukocytes from the patients during treatment with AzUR. Almost all patients typified by response A who experienced sustained inhibition of the enzymatic activity of their cells had objective improvement of their disease process. An example of this response is patient #4 with chronic granulocytic leukemia in blastic crisis, who showed an excellent depression (87 per cent) of the enzymatic activity of the leukocytes not only after the 1st day of treatment, but also on the 7th day of AzUR-therapy. These determinations were performed in cells obtained 1 hour after an injection of AzUR, 60 mg/kg. In this patient there was profound diminution in the number of circulating leukemic cells (40,000/cu mm to 1,000/cu mm), whereas the size of the spleen, as determined by palpation, was reduced approximately 50 per cent. The patients in whom the metabolic activity was only slightly depressed and at times rose to higher than normal values (response B) in general had little if any improvement in their clinical status; however, there were no
detable exceptions such as patient #3, who showed evidence of objective improvement. The majority of cases responded in a manner similar to case C (patient #9) with a marked initial decrease in metabolic capability followed by a rapid return within 7 days to normal levels of activity despite continued therapy with AzUR. This patient did not show improvement of his disease process. Experiments are in progress to attempt to suppress the evolution of the increased enzymatic activity of leukemic cells during therapy by using inhibitors of protein synthesis.

The urinary excretion patterns of orotic acid and orotidine have been measured only in a limited number of cases; a typical result is illustrated in Chart 2. More complete characterization of these peaks has been obtained by repeated crystallization of urinary orotic acid and orotidine. These compounds cannot be detected in the urine of pa-

<table>
<thead>
<tr>
<th>Patient</th>
<th>Diagnosis</th>
<th>Control (μmole C4O2/h/10⁶ WBC)</th>
<th>AzUR (5 × 10⁻⁴ M) (μmole C4O2/h/10⁶ WBC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>PCL</td>
<td>1.56</td>
<td>0.45</td>
</tr>
<tr>
<td>2</td>
<td>AGL</td>
<td>0.92</td>
<td>0.14</td>
</tr>
<tr>
<td>3</td>
<td>AML</td>
<td>0.96</td>
<td>0.66</td>
</tr>
<tr>
<td>4</td>
<td>AML</td>
<td>0.86</td>
<td>0.11</td>
</tr>
<tr>
<td>5</td>
<td>ALL</td>
<td>0.45</td>
<td>0.18</td>
</tr>
<tr>
<td>6</td>
<td>AGL</td>
<td>0.42</td>
<td>0.29</td>
</tr>
<tr>
<td>7</td>
<td>AML</td>
<td>0.58</td>
<td>0.23</td>
</tr>
<tr>
<td>8</td>
<td>AGL</td>
<td>0.30</td>
<td>0.22</td>
</tr>
<tr>
<td>9</td>
<td>CLL</td>
<td>0.16</td>
<td>0.06</td>
</tr>
<tr>
<td>10</td>
<td>CLL</td>
<td>0.12</td>
<td>0.07</td>
</tr>
<tr>
<td>11</td>
<td>CLL</td>
<td>0.12</td>
<td>0.07</td>
</tr>
<tr>
<td>12</td>
<td>CLL</td>
<td>0.08</td>
<td>0.06</td>
</tr>
</tbody>
</table>

* PCL = plasma cell leukemia; AGL = acute granulocytic leukemia; AML = acute monocytic leukemia; ALL = acute lymphocytic leukemia; CGL = chronic granulocytic leukemia; CLL = chronic lymphocytic leukemia.

Chart 4.—The metabolism of orotic acid by normal and leukemic peripheral blood cells. Activity was determined on cell suspensions (approximately 3 × 10⁷ cells per flask) as described under "Methods." X = erythrocytes; O = leukocytes; ■ = leukocytes from the same patient after 1 day of treatment with AzUR. The diagnoses are as follows: AML = acute monocytic leukemia; AGL = acute granulocytic leukemia; CGL = chronic granulocytic leukemia; ALL = acute lymphocytic leukemia; CLL = chronic lymphocytic leukemia; PCL = plasma-cell leukemia.

Chart 5.—Different types of response obtained with isolated leukocytes from patients undergoing therapy with AzUR (A = patient 4, B = patient 8, C = patient 9). The metabolic activity is expressed as a percentage of the activity before the administration of AzUR.
tients before treatment with AzUR; but, in response to therapy with this antimetabolite, as much as 8 gm. of orotic acid and 10 gm. of orotidine have been excreted in a single 24-hour period. In certain patients (2) this high urinary output of the relatively insoluble orotic acid has resulted in complications of crystalluria; however, this has not been a problem with any of the patients described in this paper. The amounts of these pyrimidine derivatives varies considerably, as indicated in Table 2. At the present time it is not possible to relate the excretion of these pyrimidine derivatives to the amount of AzUR administered or to the clinical response of the patient because of the limited number of cases studied. The interpretation of these results is influenced not only by the above two factors but also by the following parameters: (a) the volume of tumor tissue and its biochemical characteristics, (b) the balance between the metabolic conversion of AzUR to its 5'-phosphate and the urinary excretion of the analog, and (c) the capacity of the host tissues to accommodate to the metabolic block, either by accumulating excess orotic acid and orotidylic acid or by suppressing this synthesis through a feed-back mechanism. Until these factors can be evaluated, the full sign-

### TABLE 2

**Excretion Products in Urine after AzUR Therapy**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Patient</th>
<th>AzUR (gm.)</th>
<th>Orotic acid (gm.)</th>
<th>Orotidine (gm.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute monocytic leukemia</td>
<td>13</td>
<td>17</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Chronic granulocytic leukemia</td>
<td>2</td>
<td>10</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>Acute granulocytic leukemia</td>
<td>4</td>
<td>16 &lt;0.5</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>Acute granulocytic leukemia</td>
<td>14</td>
<td>12 &lt;0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Hodgkin's disease</td>
<td>15</td>
<td>9</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

The results of a typical measurement of the metabolic disposition of test doses of orotic acid-C¹⁴O₂O₂, injected intravenously into patients, is presented in Chart 6. The respiratory excretion of C¹⁴O₂ is expressed as specific activity of the barium carbonate. Calculation of the total excretion of isotope via this route from the anticipated total excretion of CO₂ indicates that approximately 65 per cent was excreted within 100 minutes before a test dose of AzUR and only 20 per cent during a similar period after this analog had been given. By co-crystallization with orotic acid and purification to constant specific activity, the major portion of the urinary radioactivity was identified as unchanged orotic acid. The results clearly demonstrate the capacity of azauridine to inhibit the conversion of orotic acid to carbon dioxide and uridine nucleotides. In a series of eleven patients, the urinary recovery of injected orotic acid before AzUR therapy ranged between 4 and 15 per cent and increased to values of 26–72 per cent after a single dose of 60 mg AzUR/kg. At the present time there is insufficient correlation between clinical response and these results to permit the use of this test as a guide to the selection of therapy. However, its possible application to the screening of patients for genetic defects in orotic acid metabolism is apparent and would be quite safe, since the radioactivity is lost before the orotic acid is incorporated into nucleic acids.

In using these tests as a guide to the selection of patients for treatment with azauridine, two points of view may be considered. First, patients who have high inherent activity in the isolated significance of the results obtained through the applications of this technic cannot be determined. This method is useful, however, as a means of evaluating different modalities of treatment in the same individual, particularly when used in conjunction with the test of the enzymatic activity of the isolated leukemic cells. This drug-induced orotic aciduria has a natural counterpart in the genetically transmitted disorder, reported by Hugeley et al. (6), in which the activities of the enzyme responsible for coupling of orotic acid with pyrophosphoryl ribose 5-phosphate and of orotidylic decarboxylase appeared to be deficient in certain members of the family of the patient (11).
leukemic cells may be assumed to be good candidates, because their neoplastic cells presumably depend heavily upon the synthesis of pyrimidines de novo. Second, patients with low activity may be chosen, because this low enzyme level may be suggestive of enhanced susceptibility to the anti-metabolites. However, this latter possibility is perhaps less likely than the former one, since it is assumed that sufficient amounts of uracil-containing metabolites may reach such cells (from dietary or nucleic acid-catabolic sources) to nullify the effects of such inhibition. Another argument in favor of treating leukemic patients with high levels of enzyme activity in their leukocytes derives from the results obtained with AzUR in normal dogs. It has been demonstrated previously that 18 mg AzUR/kg, given in three divided daily doses to normal dogs, will cause a potentially lethal depression of marrow function within 10 days, and yet ~0 or more times this dose does not appear to affect hematopoiesis in human subjects without apparent marrow disease (13). A comparison of the results presented in Chart 4 indicates that normal canine leukocytes have a much greater capacity to metabolize orotic acid than do their normal human counterparts, a result which may contribute in large part to the species differences in susceptibility to AzUR. In contrast to the measurements on leukemic cells or tumor tissues, estimation of the daily excretion of orotic acid and orotidine affords an indication of the effect of AzUR on the biosynthesis of pyrimidines by the whole body. Although the results presented in this paper do not yet provide the means of selecting patients for therapy, they do demonstrate quantitatively that the metabolic inhibition created by AzUR in man is the same as that observed in experimental animals.

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