Drug Effects on a Target Metabolic Pathway and on Mouse Tumor Growth: Azauridine and Decarboxylation of Orotic Acid-7-C\(^{14}\)*

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SUMMARY

An attempt has been made to relate the effect of a drug on a metabolic pathway, presumed to be its locus of action, to the tumor-inhibitory activity of the drug. Azauridine was administered subcutaneously to mice with five different ascites tumors, and growth was measured by total ascites cell counts. Aliquots of ascites tumor cells were incubated with orotic acid-7-C\(^{14}\), and the C\(^{14}\)O\(_2\) evolved was taken as a measure of orotidylic decarboxylase activity. Whereas graded dose-related tumor suppression was generally seen, inhibition of decarboxylation of orotic acid ordinarily did not parallel the effect on tumor growth. The basis for discrepancy is discussed. Attempts to predict relative in vivo sensitivity of the five tumors by azauridine effects on decarboxylation of orotic acid in vitro were unsuccessful.

Azauridine, the riboside of 6-azauracil, has been demonstrated by Jaffe and co-workers (5) to be an effective inhibitor of several transplantable mouse tumors. The carcinostatic properties of this antimetabolite have been attributed to an interference with the de novo synthesis of pyrimidine nucleotides by inhibition of orotidylic decarboxylase (1, 4, 8). It has been suggested that the orotidylic decarboxylase activity may have value in determining the potential chemotherapeutic efficacy of azauridine (2, 11).

Handscharmacher and Pasternak's (4) experiments with tumor slices in vitro have shown that liberation of C\(^{14}\)O\(_2\) from added orotic acid-7-C\(^{14}\) is markedly inhibited by azauridine. A schema for the sequence of reactions in this inhibition is shown in Schema 1. Orotic acid-7-C\(^{14}\) is metabolized to orotidylic acid-7-C\(^{14}\), and the activity of orotidylic decarboxylase in catalyzing the decarboxylation to uridylic acid is inhibited by azauridylic acid (4). Orotidine has been found in large amounts in azauridine-treated L5178-Y, L1210, S-180 and Ca-755 mouse tumors, but not in uninhibited tumors. This presumably arose from dephosphorylation of orotidylic acid, which was not normally decarboxylated because of the azauridine treatment (8). Thus, a critical enzyme on the pyrimidine anabolic pathway was recognized for which an effective inhibitor had been found.\(^1\)

We have studied the decarboxylation of orotic acid-7-C\(^{14}\) and growth in five transplantable mouse ascites tumors at several levels of azauridine treatment. This system of tumor treated by drug seemed well suited to determine chemotherapeutic effect, measured as growth suppression, and the inhibited biochemical pathway thought to be responsible for the activity of the drug. In addition, we have sought a correlation of enzymatic inhibition in vitro with tumor suppression in vivo in an attempt to explore the validity of predicting chemotherapeutic response from biochemical tests beforehand.

MATERIALS AND METHODS

Tumors.—In vivo experiments were performed on DBA/2 female mice given inoculations intraperitoneally of \(1 \times 10^6\) leukemia P-288 cells, or

\(^1\) A technic for the measurement of the conversion of orotic acid to uridine nucleotides was devised by members of the Department of Pharmacology, Yale University, and was kindly made available to us (S. Cardoso, R. E. Handscharmacher, and A. D. Welch. A presumptive biochemical test for tumor susceptibility to 6-azauridine. Personal communication, 1959).
2 × 10⁶ leukemia L1210 cells; Swiss Ha/ICR female mice were given inoculations intraperitoneally of 10 × 10⁶ Krebs 2 (K-2) or Sarcoma 180 (S-180) cells or 1 × 10⁶ Ehrlich (E) ascites tumor cells. Seven to ten animals were included in each treatment group.

Treatment procedure.—Starting 1 day after inoculation, 0, 50, 100, or 200 mg azauridine/kg was administered subcutaneously for 7 days, divided into morning and late afternoon doses of drug in 0.5 ml. of 0.85 per cent sodium chloride. and repeated rinsing of the peritoneal cavity, and the cells were counted.

Decarboxylation of orotic acid.—An aliquot of undiluted ascites fluid was taken and immediately centrifuged at 4°C. The cells were rapidly washed (approximately 30 seconds) with cold Krebs III physiological salt solution (6), and a 20 per cent suspension in Krebs III solution was prepared. Occasionally, cells had to be rinsed from the peritoneal cavity with Krebs III solution when the amount of ascites fluid was small. Incubations were carried out aerobically in Warburg flasks at a final volume of 3.2 ml. with constant shaking at 37°C. in the presence of 0.3 μmoles of orotic acid-7-C¹⁴ (205,000 counts/min/flask).

Incubations were started within 15 minutes after removal of the ascites fluid, and run for 60 minutes. In a preliminary experiment, decarboxylation by Krebs-2 tumor was found to be linear over a 1-hour span. Incubations were terminated by introducing HClO₄ from a side-arm of the vessel to give a final concentration of 4 per cent in the medium. The incubation flasks were then al-

* New England Nuclear Corporation.
allowed to stand 1/2 hour in an ice bath to allow for absorption of CO₂ into 2 N NaOH in the center well. Decarboxylation of the substrate was determined, after the evolution and trapping of labeled CO₂ by counting BaCO₃ with a thin-window Geiger counter. The biochemical activity measured will be described as "orotic acid decarboxylation" rather than as "orotidyl acid decarboxylase," since many intermediate steps which might have influenced the disposition of the labeled CO₂ (Schema 1) were not measured. The mmoles of CO₂ produced in incubations are expressed and interpreted per 10⁶ cells in 1 hour. Data are also presented as mmoles CO₂/mg DNA in 1 hour, since they frequently varied from the data expressed with reference to cells.

Experiments in vitro.—The relative sensitivity of orotic acid decarboxylation to azauridine in vitro was determined in five different types of mouse tumors. S-180, K-2, and E ascites cells were obtained on the 8th day, L1210 on the 6th day, and P-288 on the 4th day after implantation. All were incubated for 60 minutes in the presence of 0.05 or 0.005 mmoles of azauridine/ml of reaction mixture containing 0.5 mmoles orotic acid-7-C¹⁴ (205,000 counts/min/flask) in Krebs III buffer. Decarboxylation was determined as previously described. The results of experiments in vitro are expressed as mmoles CO₂/mg DNA in 1 hour.

DNA.—Tissue residues were extracted after incubation according to the procedure of Schneider (9). DNA determinations on the trichloroacetic acid extracts were estimated according to Seibert's modification (10) of the Dische diphenylamine reaction, with a purified DNA (Worthington) used as a standard.

Statistical methods of analysis.—Standard statistical technics were used throughout. Statements about the significance of cell counts and C¹⁴O₂ liberated were based on an analysis of their logarithms. This technic was used because the degree of variability in cell counts and in decarboxylation is closely related to the absolute levels of these determinations.

Observed values for tumor suppression or inhibition of decarboxylation are called "significant" when a value as great or greater could have occurred by chance only 1 in 20 times or less. Two levels of significance are to be distinguished: (a) significance of positive effects within a given experiment (indicated by a dagger [†] in Table 1): the basis for this significance is intraexperimental variability; (b) significance of reproducibility (indicated by a section mark [§] in Table 1): this implies not only that positive effects have been produced, but also that one may expect to reproduce these effects in future experiments under similar conditions. The basis for this significance is interexperimental variability.

RESULTS

Effect in vivo.—In Charts 1–5, the results of replicate experiments are shown. The effects of

Charts 1–5.—Effects of increasing doses of azauridine, given subcutaneously, divided into two injections daily, on decarboxylation of orotic acid-7-C¹⁴ and tumor growth in E, K-2, P-288, S-180, and L1210 ascites tumors. The ordinate scales for millions of ascite cells (□) and mmoles of C¹⁴O₂ recovered in 1 hour per million cells (□) and per milligram of deoxyribonucleic acid (■) apply to the respective values in each graph. Each control point represents mean data from ten mice, and each azauridine-treatment point, data for seven animals.
increasing doses of azauridine on total ascitic cell count are presented, and the decarboxylation of orotic acid plotted in two different ways is shown: as μmoles CO₂ liberated/1,000,000 cells in 1 hour, or per mg. DNA in 1 hour.

In three experiments (Charts 1b, 2b, 3b) cell number was suppressed approximately as much with the 50 mg. of azauridine per kg. daily dose as with the 100 or 200 mg/kg dose, thereby achieving an apparent plateau without further dose response. In all other experiments (seven charted and two others in Table 1) progressive decrease of cell number was seen with increasing dose, providing evidence of dose responsiveness in terms of tumor inhibition. The decarboxylation of orotic acid did not demonstrate this same order of dose responsiveness.

In Chart 1a, reasonable concordance is seen for E ascites tumor growth and orotic acid decarboxylation, expressed in terms of cells, but not in terms of DNA. In Chart 1b, the correlation of CO₂ liberated with the sharp decrease in cell number, at the 50 mg/kg/day dose, is poor irrespective of the reference standard.

In Chart 2a, orotic acid decarboxylation was markedly inhibited by the 50 mg/kg daily dose of azauridine. The decarboxylation activity maintains a fairly constant plateau at this low level, despite further tumor suppression with higher drug dose. In Chart 2b, the decarboxylation activity is equal at the lowest drug dose to that at the highest dose, whereas greater tumor suppression occurred at the 200 mg/kg dose level.

In Chart 3a, progressive suppression of P-288 ascites tumor growth is not matched by similar decarboxylation activity. Indeed, when expressed with respect to cell number or per mg. of DNA, decarboxylation is minimum at the 50 mg/kg/day dose level. In Chart 3b, substantial similarity of the plateau-type curves of cell number and orotic acid decarboxylation per million cells can be seen. When enzymatic activity is referred to DNA, however, there is sharp divergence between growth and enzymatic inhibition at the 50 mg/kg daily dose.

In Charts 4a and 4b, the growth of S-180 ascites tumors is progressively suppressed by increasing doses of azauridine. The decarboxylation of orotic acid, expressed in terms of cells or DNA, is marked at the 50 mg/kg dose level and fails to show dose responsiveness in either experiment.

In Chart 5a, the only significant tumor inhibition of L1210 was achieved at the 200 mg/kg daily dose. At this point, decarboxylation of orotic acid, expressed in terms of cells, is slightly higher than the control and is nearly identical per mg. of DNA. In Chart 5b, there is only suggestive concordance of inhibition of orotic acid decarboxylation per million cells and the progressive growth suppression. The enzymatic activity when expressed per mg. of DNA is strikingly discordant.

Charts of two experiments in which enzymatic activity and tumor growth were assayed are not shown (Table 1 and footnotes; experiments 1c and 5c). The decarboxylation activity of E ascites tumor was concordant and depressed to a plateau. A discordant response of growth and enzymatic activity was observed in L1210, dependent chiefly upon the data for the control group.

Table 1 summarizes the effect of azauridine on growth of the five transplantable tumors. In twelve individual experiments with five tumors, highly significant suppression (†) at the 200 mg/kg daily dose level occurred in each instant and in all but one experiment at the 100 mg/kg daily dose. In addition, the reproducibility of suppressing growth of K-2 was analyzed from both experiments and shown to be significant (§) at both the 100- and 200-mg/kg daily dose of azauridine (Experiments 2a and 2b). E carcinoma also was shown to be suppressed reproducibly at the 200 mg/kg daily dose (Experiments 1a, 1b, and 1c).

Effect in vitro.—The relative sensitivity of the decarboxylation of orotic acid in different mouse tumor cells to azauridine in vitro is demonstrated in Chart 6. At both the .05- and .005-μmole/ml level of azauridine, liberation of CO₂ by S-180 is the most completely inhibited by the compound, with K-2, E, L1210, and P-288 following in this order. At both concentrations of azauridine, the inhibition is highly significant in all five tumors. Preincubation of tumor cells with azauridine at .005 μmoles/ml for 15 minutes before addition of orotic acid-7-C¹⁴ caused only a minor change in measured enzymatic inhibition. When .05 μmoles/ml were added in similar fashion, the effect of preincubation was inconsequential.

Prediction of response.—The data obtained from the incubations in vitro of different tumor types were used in an attempt to predict chemotherapeutic response in vivo. Our initial studies in vivo involved only K-2, E, and L1210 tumors. Studies of these tumors in vitro showed that depression of enzymatic activity by azauridine was greatest for K-2, next for E carcinoma, and least for L1210. Chart 7 summarizes the correlation between experiments in vitro and chemotherapeutic effect. K-2, E, and L1210 seem to demonstrate a substantial relevance between enzymatic activity in vitro and tumor growth in vivo at two dose levels. When similar data were accumulated and used to predict the responsiveness of P-288 and S-180 tu-
TABLE 1
EFFECT OF SUBCUTANEOUS ADMINISTRATION OF AZAURIDINE ON ASCITES TUMOR GROWTH*

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>Tumor</th>
<th>None</th>
<th>50 mg/kg</th>
<th>100 mg/kg</th>
<th>200 mg/kg</th>
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<tbody>
<tr>
<td></td>
<td>Cells × 10^6</td>
<td>Cells × 10^6</td>
<td>Per cent inhibit.</td>
<td>Cells × 10^6</td>
<td>Per cent inhibit.</td>
</tr>
<tr>
<td>1a</td>
<td>E</td>
<td>535 ± 337</td>
<td>106 ± 36</td>
<td>72†</td>
<td>185 ± 46</td>
</tr>
<tr>
<td>1b</td>
<td>K-2</td>
<td>647 ± 154</td>
<td>448 ± 115</td>
<td>31</td>
<td>251 ± 60</td>
</tr>
<tr>
<td>1c</td>
<td>P-288</td>
<td>350 ± 107</td>
<td>257 ± 79</td>
<td>27</td>
<td>192 ± 44</td>
</tr>
<tr>
<td>2a</td>
<td>S-180</td>
<td>706 ± 83</td>
<td>568 ± 76</td>
<td>20</td>
<td>417 ± 56</td>
</tr>
<tr>
<td>2b</td>
<td>L1210</td>
<td>358 ± 91</td>
<td>252 ± 49</td>
<td>56†</td>
<td>291 ± 67</td>
</tr>
<tr>
<td>3a</td>
<td></td>
<td>584 ± 109</td>
<td>106 ± 36</td>
<td>72†</td>
<td>169 ± 56</td>
</tr>
<tr>
<td>3b</td>
<td></td>
<td>565 ± 56</td>
<td>106 ± 36</td>
<td>72†</td>
<td>407 ± 50</td>
</tr>
</tbody>
</table>

* The data are expressed as total number of ascites tumor cells per animal.
† Significance of effects within a given experiment. P < .05.
‡ Decarboxylation of orotic acid in mmoles CO₂/10⁶ cells/hr and in mmoles CO₂/mg DNA/hr, respectively: 0 = 200, 174; 100 = 148, 121; 200 = 150, 130.
§ Significance of reproducibility. P < .05.
# Decarboxylation of orotic acid in mmoles CO₂/10⁶ cells/hr and in mmoles CO₂/mg DNA/hr, respectively: 0 = 60, 70; 50 = 186, 139; 100 = 110, 122; 200 = 108, 103.

<table>
<thead>
<tr>
<th>CONTROL</th>
<th>0.005 mmoles AZUR</th>
<th>0.05 mmoles AZUR</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-180</td>
<td>65%</td>
<td>58%</td>
</tr>
<tr>
<td>K-2</td>
<td>58%</td>
<td>49%</td>
</tr>
<tr>
<td>E</td>
<td>55%</td>
<td>37%</td>
</tr>
<tr>
<td>L1210</td>
<td>45%</td>
<td>55%</td>
</tr>
<tr>
<td>P-288</td>
<td>42%</td>
<td>29%</td>
</tr>
</tbody>
</table>

CHART 6.—Effect of azauridine in vitro on decarboxylation of orotic acid-7-C₁⁴ by different mouse ascites tumors. Percentage inhibition by 0.05 and 0.005 mmoles azauridine/ml of reaction mixture is indicated above each bar. S-180 and K-2 are combined data from two experiments each, and E from five experiments. When the treated values are contrasted with their respective controls in each tumor at each dose, P is < .001 that the results could have occurred by chance.

CHART 7.—Relation of in vitro effects of azauridine plotted on abscissa to in vivo effects plotted on ordinate in different mouse ascites tumors. Encircled letters, e.g., (7), indicate the tumor type incubated in vitro with azauridine at 0.005 mmoles/ml and treated in vivo at 100 mg/kg/day. Ensquared letters, e.g., [K], indicate the tumor type incubated with azauridine at 0.05 mmoles/ml and treated in vivo with 200 mg/kg/day.
mors, however, the inhibition of enzymatic activity by azauridine treatment in vitro was grossly discordant with the relative growth suppression in vivo.

DISCUSSION

That azauridine interferes in some way with the metabolism of orotic acid has been well established by several investigators (2-4, 7, 8). Pasternak and Handschumacher demonstrated a marked inhibition of orotic acid incorporation into nucleic acids of various normal mouse tissues by azauridine (8) and accumulation of large amounts of orotidine in tumors of azauridine-treated mice (7, 8). They have proposed that azauridine is phosphorylated to azauridylic acid, at which level it interferes with the decarboxylation of orotidylic acid (Schema 1). In earlier studies, Jaffe and others (5) had shown that azauridine is a potent inhibitor of the growth of certain transplantable mouse tumors.

The present investigation was carried out to study simultaneously the effect of a drug on tumor growth and on a target pathway, susceptible to the drug's action through which the tumor-inhibitory effect was presumed to be exercised. By observation of appropriate correlations, it was hoped that one could define whether the tumor inhibition had occurred concurrently with or dependent upon the measured biochemical effect.

The systems we have used may be criticized for several inherent potential sources of error. We have assumed that none of the steps in the metabolism of azauridine to azauridylic acid, nor in the absorption of or conversion of orotic acid to orotidylic acid, is rate-limiting. It is possible that, in certain circumstances, one of these steps might be rate-limiting, and that the sum effect, measured as decarboxylation of orotic acid, might be dependent on unknown variables. In the course of metabolism of orotic acid to uridine nucleotides, by a supernatant fraction of rat liver homogenate, no detectable orotidylic acid appeared. When uridine 5'-phosphate was added, however, it (or its metabolic products) exerted strong inhibitory effects on the disappearance of the orotidylic acid formed, which led to its accumulation (1). It is conceivable, therefore, that endogenous levels of uridine nucleotides in the tumor cells we studied might influence the rate of orotidylic acid decarboxylation. Similarly, azauridine effects on normal tissues might modify the pool of uridine compounds exogenously available to the tumor cells. We measured enzymatic activity by the extracellular trapping of C^4O_2. Intracellular diversion of C^4O_2 to other metabolic pathways would give falsely low values for decarboxylation of orotic acid.

Considerable variation is seen in most experiments when one compares the enzymatic activity expressed per unit of cells with that per unit of DNA. No certain explanation for this can be advanced. It can be seen that in most experiments decarboxylation of orotic acid per mg. of DNA is higher in the groups treated with the 200 mg/kg/day dose than with the 50 mg/kg/day dose. This need not reflect a change in enzymatic content, but perhaps a lower DNA concentration per cell. More nearly complete interference with accumulation of DNA during interphase at the higher dose could be an explanation for the observed effects.

The ascites cell counts have been uncorrected for contamination by leukocytes and other normal host cells. Lastly, it is certain in L1210 and P-288, and probably in the other neoplasms, that the peritoneal content of ascites cells was not the whole population of tumor cells. The effects which migration of leukemic cells to and from the peritoneal cavity might have on enzymatic activity and observed tumor inhibition are unknown.

In the five tumors studied—E, K-9, P-288, S-180, and L1210—suppression of tumor growth by azauridine was substantial and statistically significant at the 100 and 200 mg/kg/day dose levels. In seven instances presented graphically, graded dose response was observed, and in three other experiments (Charts 1b, 2b, and 3b) a near-maximum effect occurred with the lowest dose of azauridine administered without important change as dose increased. Concordance of enzymatic inhibition and effect on growth occurred most convincingly when the growth curves demonstrated this apparent plateau effect (Charts 2b, 3b). There is a suggestion of graded inhibition of decarboxylation of orotic acid per million cells and of total cellular growth in two experiments (Charts 1a and 5b). In the other experiments, however, where enzymatic activity is expressed either with reference to cells or to DNA, the evidence for causal relationship in this system is not persuasive.

The possibility exists that drug effects on enzymatic activity and total ascitic cell population might apparently be dissociated if the most susceptible cells had been eliminated. In such a circumstance the relatively more resistant cells capable of surviving to the 8th day after start of treatment might contain enzyme, even in supra-normal amounts, unaffected by the drug. This hypothetical explanation might be compatible with results such as those shown for L1210 in Charts 5a
and 5b but would not explain the dissociated effects seen, for example, in Charts 2a and 4a.

The control groups of the several tumors show a varying activity for decarboxylation of orotic acid per million cells and per mg. of DNA among tumor types (contrast S-180 experiments, 4a and 4b, with the four other tumors); and even among different passages of the same tumor (compare the two P-288 experiments, 3a and 3b). There is no apparent relation of this variability in control enzymatic activity to control tumor growth, nor to tumor response of the azauridine-treated groups. In the K-2 tumor nearly complete inhibition of decarboxylation of orotic acid occurred (Charts 2a and 2b). In E tumor, starting at approximately the same enzymatic activity (Chart 1a), the enzymatic suppression was considerably less, although the growth inhibition was indistinguishable from that of K-2. In S-180 (Chart 4a), and P-288 (Chart 3b), decarboxylation of orotic acid per cell was as high as any encountered. Enzymatic inhibition from treatment was incomplete, however, with residual activity in the azauridine-treated groups at a decreased but still substantial level. Nonetheless, growth suppression was pronounced.

These data on the several tumor experiments exemplify the complexity of validating a biochemical basis for drug action against a tumor in vivo.

The validity of predicting chemotherapeutic response from a biochemical assay was tested in the present studies. It was hoped that it might be possible to rank the order of susceptibility of different tumors to the action of azauridine in vivo from studies of enzymatic inhibition by the drug in vitro. The in vitro enzymatic inhibition and in vivo tumor suppression for two of five of the tumors tested was not apparently correlated. In no instance was the aliquot of the pooled ascites tumor on which in vitro assays were made also used as the inoculum for an in vivo experiment. Thus, all in vitro experiments performed for prediction purposes are at least one transplant generation removed from the experiments in which an attempt was made to validate the prediction. Although five in vitro experiments with E carcinoma over an extended span were highly reproducible, the different enzymatic activities in different transplant generations of P-288 (Charts 3a, 3b) demonstrate that variability can occur. Furthermore, the decarboxylation of orotic acid per mg. DNA in 1 hour for the control groups in the in vitro experiments, is, in general, less than the activity measured in the in vivo experiments. It is possible that such variability was operative in those experiments where poor chemotherapeutic predictions for P-288 and S-180 were obtained. From the data available, however, one cannot substantiate this, and the validity of the prediction technic remains unproved.

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