Arabinosyl-5-azacytosine: Mechanisms of Native and Acquired Resistance

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ABSTRACT

Factors influencing the activity of the nucleoside analogue arabinosyl-5-azacytosine (ara-AC) were studied in P388 murine lymphoblasts in vitro and in vivo, in variants of these cells with artificially acquired resistance, in the naturally resistant colon 38 carcinoma in vivo, and in a panel of six human tumors maintained in continuous culture. Differences were noted not only between the sensitive and artificially developed resistant variants of P388, but also between the naturally sensitive (P388) and naturally resistant (colon 38) tumors. The artificially developed resistant P388 cell lines showed an inhibited capacity to accumulate nucleotides derived from ara-AC and deoxyctydine, whereas the accumulation of cytidine nucleotides remained unchanged. Studies of the initial velocity of facilitated diffusion of ara-AC showed only minor differences between parental and resistant lines, while the nucleotide formation rates from both ara-AC and deoxycytidine were markedly depressed in the latter cells. It is concluded, therefore, that the failure of resistant P388 cells to accumulate these compounds results not from a transport defect per se but rather from a failure to convert the nucleosides to nondiffusible (i.e., phosphorylated) species inside the cell. This failure was accompanied by a substantial reduction in the incorporation of a radioactively labeled product derived from deoxyctydine into the nucleic acids of the resistant clones. The common factor responsible for the resistance of P388 variants toward ara-AC appears to be a markedly decreased level of deoxycytidine kinase activity. The naturally resistant colon 38 carcinoma, on the other hand, in addition to a decrease in the activity of its deoxycytidine kinase, showed a lower level of activity of all its purine and pyrimidine kinases, along with a notably elevated nucleoside triphosphate accumulation in colon 38 than in P388 after comparable drug exposure. In the six human tumor lines, a positive correlation was established between sensitivity to ara-AC (as determined by its median inhibitory concentration) and cellular content of deoxycytidine kinase. It is concluded that this latter enzyme is a generally important determinant of sensitivity to arabinosyl-5-azacytosine.

INTRODUCTION

In previous reports (1, 2) we documented that the nucleoside analogue ara-AC4 exhibits an unusual spectrum of activity against transplantable tumors, including 3 human xenografts (MX-1, LX-2, and CX-1) growing in athymic mice, and that the drug undergoes extensive intracellular phosphorylation but limited incorporation into the DNA of susceptible cells. Also described was the observation that, despite the breadth of the chemotherapeutic activity of ara-AC, not all transplantable tumors are responsive to the drug, colon 38 carcinoma being a notable case in point (1). In the present paper, an attempt has been made to determine the basis for such natural resistance to ara-AC and to compare the factors operative in the case of colon 38 with those underlying the resistance of variant lines of P388 cells selected either by exposure in vitro to incremental concentrations of ara-AC or by chemical induction of mutation followed by cultivation in the presence of high concentrations of the drug. Finally, the contribution of deoxycytidine kinase to the responsiveness of a panel of cultured human cancers has been examined in vitro.

MATERIALS AND METHODS

Materials

[6-3H]ara-AC (10.9 Ci/mmol) and [2,4-14C]ara-AC (16.8 mCi/mml) were obtained from Research Triangle Institute (Research Triangle Park, NC). [8-14C]Adenosine (54 mCi/mmol), [2-14C]Juridine (53 mCi/mmol), and [U-14C]Thymidine (458 mCi/mmol) were purchased from Amersham Corporation (Arlington Heights, IL). [2-14C]Deoxycytidine (25 mCi/mmol), sodium [14C]Carbonate (50 mCi/mmol), and [2-14C]-Thymidine (51.6 mCi/mmol) were purchased from New England Nuclear (Boston, MA). [8-14C]ATP (44 mCi/mmol) was obtained from Schwarz/Mann (Spring Valley, NY). [5-3H]dCMP (22 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). ara-AC, ara-C, and AC were obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute, NIH (Bethesda, MD). Nonlabeled nucleosides were obtained from Sigma Chemical Co. (St. Louis, MO). Enzymes were purchased from Sigma or Boehringer. All other reagents were of the highest quality obtainable.

Development of Resistant Cell Lines in Vitro

Drug-induced Resistance. P388 lymphoblasts were grown in RPMI 1640 medium containing 10% calf serum, 2 mM L-glutamine, and 5 µM 2-mercaptoethanol. The sensitivity of this line to ara-AC was determined as described previously (1). ara-AC-sensitive P388 lymphoblasts (IC50 = 2 µM) were rendered resistant to the drug by stepwise increases of the ara-AC concentration from 1 µM to 4 mM, using a procedure analogous to that described previously (3). This procedure resulted in a resistant line capable of tolerating drug levels greater than 2000 times the IC50 value of the parent line. This line maintained stable resistance, even when cultivated for over 100 generations in the absence of the drug; it exhibited a doubling time (12–14 h) similar to that of the parental strain.

Resistance Induced by Chemical Mutagenesis and Selection of Mutants by Cloning. P388 cells, at a density of 5 x 105 cells/ml in complete RPMI 1640 medium, were exposed to MNNG (1.5 µg/ml) for 3 h. The cells were collected by centrifugation, followed by resuspension in fresh complete medium, and then maintained under nonsel ective growth conditions for 10 days to allow potential phenotypic expression. ara-AC, at a concentration of 50 µM, was added to the cell population fresh daily for 2 days. The cells were then suspended at a density of 106 cells/ml in cloning medium (RPMI 1630 medium containing 20% fetal bovine serum, 50 µM 2-mercaptoethanol, 0.1% Noble agar, and gentamicin, 50 µg/ml) containing 50 µM ara-AC and incubated at 37°C in capped culture tubes. After 14 days, eight different clones of these cells were isolated and allowed to grow separately in complete RPMI 1640 medium. Cytotoxicity toward ara-AC was determined using growth rate curves as described previously (1). The eight resistant clones exhibited doubling times roughly comparable to those of the parent line. These clones were cultured in RPMI 1640 medium containing 10% calf serum, 2 mM L-glutamine, and 5 µM 2-mercaptoethanol. The sensitivity of this line to ara-AC was determined as described previously (1). ara-AC-sensitive P388 lymphoblasts (IC50 = 2 µM) were rendered resistant to the drug by stepwise increases of the ara-AC concentration from 1 µM to 4 mM, using a procedure analogous to that described previously (3). This procedure resulted in a resistant line capable of tolerating drug levels greater than 2000 times the IC50 value of the parent line. This line maintained stable resistance, even when cultivated for over 100 generations in the absence of the drug; it exhibited a doubling time (12–14 h) similar to that of the parental strain.

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3 The abbreviations used are: ara-AC, 1-β-D-arabinofuranosyl-5-azacytosine; ara-C, 1-β-D-arabinofuranosylcytosine; AC, 5-azacytidine; IC50, median inhibitory concentration; NBMPR, nitrobenzylthioinosine; DTT, dithiothreitol; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; and TCA, trichloroacetic acid.
resistant lines were arbitrarily labeled P388/R-1 to P388/R-VIII (This nomenclature will be used throughout the text). The sensitivity of these lines toward structural analogues of ara-AC, i.e., ara-C and AC, was also determined in side-by-side experiments.

**In Vivo** Tumors. P388 leukemia and colon 38 carcinoma were grown s.c. in C57BL/6 × DBA/2 (hereafter called B6D2F1) mice as described in a previous report (1). The generation time of P388 tumors was 12–14 h, and that of the colon 38 tumors was approximately 2 days.

Uptake Studies. Uptake studies were carried out by a modification of the method used by Wiley et al. (4) to study the influx of ara-C. P388 lymphoblasts or variant cell lines growing in log phase at a cell concentration of 2–5 × 10^7/ml were centrifuged at 1000 × g for 5 min. The resultant cell pellet was resuspended in complete RPMI 1640 medium, at a concentration of 8–10 × 10^6 cells/ml. A 500-µl aliquot of this cell suspension was treated with 1.21, 1.48, or 1.65 µCi of radiolabeled ara-AC, deoxycytidine, or cytidine, respectively, at a final concentration of 5 µM. Cells were allowed to incubate at 37°C for 1–15 min, over 0.75 ml of Versilube in microcentrifuge tubes, and then centrifuged for 30 s at 12,500 × g. After centrifugation, the tubes were placed on dry ice to freeze the upper medium layer and the cell pellet. Using an electrically heated red-hot wire, the tubes were cut and the cell pellet separated from the medium. This pellet was allowed to digest overnight at 37°C in 0.5 ml of 1 N NaOH. The digested pellet was neutralized with 0.5 ml of equimolar HCl and radioactivity determined using scintillation spectrometry.

For studies of the initial velocity of uptake, P388 cells, 3–6 × 10^7/ml, were exposed to 20 µM nitrobenzylthioinosine [NBMPR, a nucleoside influx inhibitor (4)] or saline for 1 h before the addition of 5 µM [14C]ara-AC. After 30 s at 37°C, the cells were centrifuged through Versilube and processed as described above. The rate of facilitated diffusion so measured was taken as the difference in uptake between cells exposed to saline versus NBMPR.

**Metabolism**

**In Vitro.** Sensitive and resistant cell lines were exposed to labeled ara-AC, deoxycytidine, or cytidine as described earlier. Conditions for the incubation and centrifugation were identical to those described above except that the still-frozen cell pellet was vigorously vortexed in 10% TCA. The acid-soluble fraction was isolated by centrifugation at 12,500 × g for 2 min and neutralized with 0.5 M triocetylamine in freon (5). Neutralized samples were loaded onto a Partisil-10 SAX column (Waters Associates, Milford, MA) preequilibrated with 0.03 M ammonium acetate [NMBPR, a nucleoside influx inhibitor (4)] or saline for 1 h before the addition of 5 µM [14C]ara-AC. After 30 s at 37°C, the cells were centrifuged through Versilube and processed as described above. The rate of facilitated diffusion so measured was taken as the difference in uptake between cells exposed to saline versus NMBPR.

**Isopycnic Centrifugations.** P388/S and P388/R cells in logarithmic growth were incubated for 5 h with 5 µM [14C]ara-AC, [14C]cytidine, or [14C]deoxycytidine and pelleted by low-speed centrifugation. The pellets were washed twice with cold phosphate-buffered saline and twice with 60% ethanol, then redissolved in a 100-µl lysis buffer consisting of Tris-HCl, 10 mM, pH 7.3; NaCl, 100 mM; EDTA, 1 mM; sodium dodecyl sulfate, 0.2%; and N-laurylsarcosine 0.5%. The lysate was layered over a solution of cesium chloride (1.71 g/ml) in 4.4 ml Beckman polyanal- lomer tubes and centrifuged for 72 h at 30,000 rpm in a Beckman SW56 rotor. The tubes were punctured with a stainless steel needle from the bottom, and the distribution of radioactivity in RNA (p > 1.85) and DNA (p = 1.70–1.71) determined by scintillation spectrometry (8).

**Detection of Radiolabeled Formate.** ara-AC decomposes in two steps to yield formic acid; in the case of [6-3H]-ara-AC this formate will be radioactive. Radiolabeled formate was separated from tritiated ara-AC and its ring-opened form by chromatography on a 1-×20-cm column of HAX4 resin (formate form), developed with a linear gradient of water (250 ml) to 1 M formic acid (250 ml), at a flow rate of 1.2 ml/min. Under these conditions, parent drug eluted in the void volume (1–2 min), followed by the ring-opened species (5 min), and the formate eluted at 90 min.

**Nucleotide Determination**

Groups of 5 male B6D2F1, mice, bearing s.c. implanted P388 or colon 38 tumors, about 1 cm in diameter, were killed by cervical dislocation, and the tumors were excised, and immediately flash frozen between blocks of dry ice. The sample preparation and HPLC analysis methods used were similar to those described above for in vitro metabolism.

**Measurements of Nucleoside Kinases**

P388 cells in culture or freshly excised tumor nodules were homogenized in 15°C buffer containing 0.1 M Tris-HCl and 1 mM DTT, pH 8.0, using a Polytron homogenizer. Homogenates were centrifuged at 12,500 × g for 2 min and the supernatants were used as an enzyme source. Nucleoside kinases were assayed by modification of the procedure described by Anderson (9) for uridine-cytidine kinases. For analysis of kinase activities, 5 µl of 1 mM [14C]nucleoside precursor (50 µCi/ml) and 5 µl of 30 mM ATP-MgCl2 (in 0.1 M Tris, pH 7.8) were added to 1.5-ml Eppendorf vessels. The reaction was initiated by the addition of 5 µl of the enzyme extract or buffer blank. After incubation at 37°C for 30 min, the reaction was stopped by heating at 95°C for 2 min. The vessels were centrifuged at 12,500 × g for 2 min, and an aliquot of the sample was spotted on Whatman 3MM paper for ascending chromatography, using ethanol:1 M ammonium acetate (7:3) as solvent, or on PEI cellulose plates, using 0.2 M KH2PO4 at native pH as solvent (10). Nucleotide spots were excised, eluted with 1 ml of water, and counted by scintillation spectrometry.

**Measurement of 5'-Ectonucleosidase**

5'-Ectonucleosidase (EC 3.1.3.5) was measured by a modification of the method of Price (11). The assay was performed by incubating 25 µl (3–4 × 10^4 cells in Hanks' balanced salt solution, pH 7.4) of each of the nine P388 lines (P388/R1-VIII and P388/S) with 5 µCi of dried [3H]dCMP (adjusted with cold dCMP to a final specific activity of 500,000 cpm/ml) at 37°C for 20 min. The reaction was then terminated by heating the vessels for 1 min at 95°C and centrifuging briefly to remove the precipitated proteins. Five µl of the supernatants were then spotted on Whatman 3MM paper and chromatographed overnight in a solvent mixture of 1 M ammonium acetate (native pH):absolute ethanol, 30:70 (v/v). Spots corresponding to authentic deoxycytidine were excised and radioactivity determined by scintillation spectrometry.

**Measurement of Nucleoside Triphosphatase**

Tumors or cell pellets were homogenized in cold 0.1 M Tris-HCl, pH 7.8, containing 1 mM DTT. The homogenates were centrifuged at 12,500 × g for 5 min, and supernatant fractions were dialyzed against Tris-DTT buffer for 3 h. ATP breakdown was measured in a reaction
mixture containing [14C]ATP (7.5 mM, 0.33 μCi/μmol), MgCl2 (7.5 mM), and crude extract in a final volume of 20 μl. Incubation was carried out in Eppendorf tubes for 30 min at 37°C, and the reaction was terminated by heating at 95°C for 1 min. After centrifugation, 10-μl aliquots of the reaction mixture were spotted on PEI-cellulose plates and overspotted with a mixture of AMP, ADP, and ATP (3 mM each). Chromatograms were developed in 0.2 M KH2PO4 (pH native). Nucleotides spots were excised and eluted with water, and radioactivity was determined by scintillation spectrometry.

Deamination of ara-AC. Freshly removed nodules of the P388 and colon 38 tumors were homogenized in 0.1 M Tris HCl, 1 mM DTT, pH 7.4, and the 105,000 × g supernatant from this step was dialyzed against a large excess of the homogenization buffer. For the conduct of the assay, 10 μl of the dialyzed enzyme was incubated with 50 μl of 10 mM ara-AC in 0.1 mM sodium phosphate, pH 7, at 37°C for 15 min. The reaction was terminated by 2 min of heating at 95°C. Coagulated proteins were removed by centrifugation, and ammonia in the supernatant was assessed enzymatically with L-glutamate dehydrogenase (12). In some cases, the generation of deaminated products was checked by reversed-phase HPLC (7).

Deoxycytidylate Deaminase. Freshly removed nodules of s.c. P388 and colon 38 tumors were homogenized in cold 0.01 M Tris-HCl, pH 8.0, containing 15% glycerol and 1 mM DTT. The homogenates were centrifuged at 105,000 × g for 1 h and the resulting supernatant used as enzyme source. For the conduct of the assays, 20 μl of crude supernatant were incubated with 100 μl of 0.01 M deoxycytidyl acid in 0.02 M Tris-HCl, pH 7.8, at 37°C for 15 or 30 min. The reaction was stopped by a 2-min heating at 95°C and denatured protein removed by centrifugation at 12,500 × g for 3 min. Five μg of alkaline phosphatase were then added to each vessel and the hydrolysis of nucleotides allowed to proceed at 37°C for 3 h. Deoxyuridine was then quantitated by reversed-phase HPLC (7).

Colony Formation and Growth Rate Measurements with Cultured Cells

Monolayers. The human lung cancer cell lines NCI-H322 and NCI-H358 were originated from bronchioloalveolar carcinoma of the lung, while the NCI-H460, tested at early and late passages (11 and 90, respectively) originated from a large-cell carcinoma. All three lines were maintained as monolayer cultures in RPMI 1640 medium supplemented with 10% fetal bovine serum. Cultures were maintained in humidified incubators at 37°C in a 5% CO2 atmosphere. The cytotoxicity of ara-AC was determined by a quantitative colony-forming assay based on the inhibition of cell proliferation. Cells were plated at a density of 20 cells/cm² in 60-mm plastic Petri dishes. After a 24-h attachment period, the cells were treated with the indicated concentration of ara-AC (or saline) and incubated with the drug for 4 days, after which the medium was removed and the cells incubated in fresh medium for an additional 5 days. Cell colonies were then fixed in methanol and stained with Giemsa. The total number and size of the stained cell colonies were determined by means of an Artrek Model 880 automatic colony counter (Artrek Systems, Farmingdale, NY).

Suspension Cultures. In order to determine the effect of ara-AC concentration on the growth rate of human and murine cells in suspension culture, the following five lines were used: P388, murine lymphoblast/macrophage leukemia; L1210, murine lymphoblast leukemia; HL-60, human promyelocytic leukemia; THP-5, human null cell leukemia; and Jurkat, human T-cell leukemia. Cell lines were maintained as suspension cultures in RPMI 1640 (with the exception of L1210, which was grown in RPMI 1630) supplemented with 10% fetal bovine serum (10% donor calf serum and 5% MRC mercaptoethanol for P388). Lines were grown in a humidified atmosphere of 5% CO2 in air, at 37°C. All suspensions were treated with ara-AC in saline or saline alone. The cell count was taken after 24 h of incubation, the growth rate was plotted against drug concentration, and the IC50 values were obtained as described previously (1).

RESULTS

Characterization of Resistance. Cell lines resistant to ara-AC were developed by the two different methods described in "Materials and Methods." Cultivation of P388 lymphoblasts in the presence of increasing concentrations of ara-AC over 100 generations resulted in a resistant variant capable of tolerating levels of the drug 2000 times higher than those tolerated by the parental strain. Resistant cell lines developed by initial exposure to the mutagenic agent MNNG, followed by cloning in the presence of a high ara-AC concentration, resulted in eight different strains exhibiting IC50 values greater than 1000 times that shown by the parental strain (Table 1). A similar cloning efficiency (approximately 80%) was observed for both the sensitive and resistant (RI-VIII) cell lines.

Also listed in Table 1 are the median inhibitory concentrations toward these cells of ara-C and AC, nucleosides congenic to ara-AC. It can be seen that the parental P388 lymphoblasts were about 40-fold more sensitive to ara-C than to ara-AC. However, ara-AC and AC were roughly equivalent in inhibiting the growth rate of P388 cells. The IC50 values of ara-C versus the cell lines resistant to ara-AC were found to be in the range of 160-1,200 μM, 3,200-24,000 times higher than the IC50 value of this agent for the sensitive cell line, a result which exemplifies the extreme resistance of these variants. On the other hand, the IC50 values of AC toward these lines indicated negligible cross-resistance to this ribonucleoside.

Uptake Studies. To initiate an investigation into the factors involved in resistance to ara-AC, the rate of accumulation of radioactivity derived from the drug was measured in the parental strain, as well as in the chemically induced resistant cell lines, and was compared with the accumulation of the natural nucleosides deoxycytidine and cytidine. The results of these studies are presented in Table 2. Total accumulation of radioactivity derived from ara-AC and deoxycytidine over a 15-min period was found to be markedly depressed in the resistant as compared to the sensitive lines. On the other hand, accumulation of radioactivity derived from cytidine was found not to be affected by the sensitive or resistant nature of these lines. It is also important to note that, in these 15-min incubations, the extent of accumulation of cytidine was substantially higher than that of deoxycytidine or ara-AC in the sensitive P388 lymphoblasts.

In order to investigate the possibility that a transport deficit for ara-AC might be operative in the resistant cells, the initial velocity of accumulation of labeled ara-AC was determined in the presence or absence of NBMPR using a 30-s incubation. The difference in uptake of ara-AC at this time in the presence or absence of the transport inhibitor was taken as an index of the rate of facilitated diffusion of the drug (4). Under these conditions, as shown in Table 2, the IC50 value of this agent for the sensitive cell line, a result indicated negligible cross-resistance to this ribonucleoside.

Table 1. Sensitivity of P388 lymphoblasts and variant lines toward ara-AC, ara-C, and AC.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>ara-AC IC50 (μM)</th>
<th>Fold resistance</th>
<th>ara-C IC50 (μM)</th>
<th>Fold resistance</th>
<th>AC IC50 (μM)</th>
<th>Fold resistance</th>
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</thead>
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<tr>
<td>P388/S</td>
<td>1.90</td>
<td>1.05</td>
<td>2.20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P388/resistant</td>
<td>&gt;4000</td>
<td>&gt;2000</td>
<td>7000</td>
<td>5.0</td>
<td>2.27</td>
<td></td>
</tr>
<tr>
<td>P388/R-I</td>
<td>&gt;2000</td>
<td>&gt;1000</td>
<td>12000</td>
<td>10.0</td>
<td>4.54</td>
<td></td>
</tr>
<tr>
<td>P388/R-II</td>
<td>&gt;2000</td>
<td>&gt;1000</td>
<td>2000</td>
<td>5.0</td>
<td>2.27</td>
<td></td>
</tr>
<tr>
<td>P388/R-III</td>
<td>&gt;2000</td>
<td>&gt;1000</td>
<td>3500</td>
<td>6.0</td>
<td>2.70</td>
<td></td>
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<tr>
<td>P388/R-IV</td>
<td>&gt;2000</td>
<td>&gt;1000</td>
<td>3500</td>
<td>6.0</td>
<td>2.70</td>
<td></td>
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<tr>
<td>P388/R-V</td>
<td>&gt;2000</td>
<td>&gt;1000</td>
<td>3200</td>
<td>6.0</td>
<td>2.70</td>
<td></td>
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<td>4.0</td>
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<td>P388/R-VII</td>
<td>&gt;2000</td>
<td>&gt;1000</td>
<td>2600</td>
<td>7.0</td>
<td>3.20</td>
<td></td>
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<tr>
<td>P388/R-VIII</td>
<td>&gt;2000</td>
<td>&gt;1000</td>
<td>2800</td>
<td>6.0</td>
<td>2.70</td>
<td></td>
</tr>
</tbody>
</table>

* P388/S, parent line, sensitive to ara-AC; P388/resistant, resistance induced by cultivation in the presence of incremental drug concentration; P388/R-I-VIII, MNNG-induced resistance, followed by cloning in 50 μM drug.
conditions, the sensitive cells accumulated ara-AC at the rate of 4.2 pmol/10⁶ cells/min; the rates measured in the eight resistant lines were slightly but insignificantly higher than this value (Table 2).

**Metabolism Studies.** To determine whether the resistant cells differed in their capacity for nucleotide formation, the in vitro anabolism of ara-AC, deoxycytidine, and cytidine was compared in the sensitive and resistant cell lines. As shown in Table 3, P388 lymphoblasts metabolized ara-AC to its mono-, di-, and triphosphates and also actively phosphorylated the natural nucleosides; however, the rate of cytidine metabolism was much higher than that of ara-AC or deoxycytidine. In most cases nucleoside triphosphates were also found to predominate over the mono- and diphosphates.

In the resistant cell lines, the anabolism of ara-AC as well as deoxycytidine was found to be greatly retarded; however, the conversion of cytidine to nucleotides was unimpaired (Table 3). These results are compatible with the conclusion that ara-AC and deoxycytidine utilize the same anabolic pathway, and that the latter is impaired in the resistant cells, while cytidine is converted to its nucleotide forms by an independent route. Thus, these in vitro metabolic studies correlate well with the uptake studies and suggest that the lack of metabolism of ara-AC and deoxycytidine to their respective nucleotides by the resistant cell lines is responsible for the deficit in their long-term (15 min) accumulation shown in Table 2.

With isopycnic density centrifugation in cesium chloride, it was established that the incorporation of radioactivity derived from [³⁴C]ara-AC into nucleic acids was at the lower limits of detectability in the resistant clones, as well as in the parental P388/S cells (<0.25 pmol/10⁶ cells) (Table 3). Incorporation of [³⁴C]ara-AC into DNA was less than 0.5% of that seen with [³⁴C]deoxycytidine under comparable conditions.

**In Vivo Studies.** In order to determine whether naturally drug-resistant tumors also lack the capacity to anabolize ara-AC, we measured the metabolism of the drug in sensitive P388 tumors and naturally resistant colon 38 tumors, both implanted s.c. The results of measurements of the anabolism of the drug to its mono-, di-, and triphosphorylated forms are presented in Table 4, where it can be seen that both lines converted ara-AC to the nucleotide level (denoted Σara-ACXP in Table 4), but that P388 lymphoblasts, grown in vivo, catalyzed this process at a rate 3–5 times that of colon 38 carcinoma. Accumulation of parent drug was very similar in both tumor types, even over a 10-fold difference in dose. Although colon 38 tumors accumulated reduced amounts of mono- and diphosphates as compared to P388 tumors, the differences remained relatively small. On the other hand, marked differences were observed in the accumulation of ara-AC-triphosphate: P388 tumors accumulated 10–20-fold higher levels of this nucleotide than the colon 38 tumors. This resulted in the incorporation of drug-associated radioactivity into TCA-insoluble material (nucleic acids), found to be approximately 4 times higher in P388 than in the resistant colon 38.

Because the triazine ring of ara-AC is known to be labile (13), the nature of the metabolites formed in these two lines was examined more closely. Toward this end, animals bearing P388 and colon 38 s.c. tumors were given [6³H]-labeled ara-AC i.p. and, after extraction, the acid-soluble pools and the nucleotides generated by DNase-phosphodiesterase digestion of the acid-insoluble residues were treated with purified bacterial alkaline phosphatase and then reexamined by reversed-phase HPLC. The results of these studies are displayed in Fig. 1, where it can be seen that, in addition to ara-AC (elution time, 11 min) and its ring-opened form (elution time, 9 min), both lines contained small amounts of radiolabeled guanosine and adenosine. Analysis of the acid-soluble pools (not shown) also revealed a large radioactive peak coeluting with formate on ion-exchange chromatography (cf. methods). These results support the contention that ring-opened ara-AC is chemically cleaved in vivo (as it is in vitro) to yield formate, which can then be utilized for the assembly of the purine ring. Analysis of the alkaline phosphatase digest of acid soluble pools (Fig. 1, A and B) confirmed our previous results (Table 4), and showed that the ara-AC-associated radioactivity is approximately 4-fold higher in P388 than in colon 38. Similar results were observed for the nucleic acid digests (Fig. 1, C and D). It is important to point out that the formate arising from the decomposition of

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**Table 2. Accumulation of radioactivity derived from ara-AC or its congeners by lymphoblasts sensitive or resistant to the drug.**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Nucleoside</th>
<th>Rate of net accumulation</th>
<th>Transport by facilitated diffusion*</th>
<th>Mean of individual cell lines</th>
<th>Incorporation into DNA (pmol/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P388/S</td>
<td>ara-AC</td>
<td>4.2 ± 0.8</td>
<td>27.0 ± 0.7</td>
<td>20%</td>
<td>0.7 ± 0.1</td>
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<tr>
<td></td>
<td>Deoxycytidine</td>
<td>ND</td>
<td>38.5</td>
<td></td>
<td>5.2 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Cytidine</td>
<td>ND</td>
<td>124.6</td>
<td></td>
<td>5.2 ± 0.8</td>
</tr>
<tr>
<td>P388/RI-VIII*</td>
<td>ara-AC</td>
<td>5.2 ± 0.8</td>
<td>3.3 ± 0.3</td>
<td>30%</td>
<td>10.6 ± 1.0</td>
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<tr>
<td></td>
<td>Deoxycytidine</td>
<td>ND</td>
<td>&lt;0.01</td>
<td></td>
<td>10.6 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>Cytidine</td>
<td>ND</td>
<td>106.0 ± 10.8</td>
<td></td>
<td>106.0 ± 10.8</td>
</tr>
</tbody>
</table>

* For transport studies, logarithmically growing P388 cells were exposed to 20 μM NBMPR or saline for 1 h before the addition of [³⁴C]ara-AC, 5 μM. At the end of a 30-s incubation with the drug, the cells were centrifuged through Versilube. The resultant pellet was digested and counted as described in "Materials and Methods." Logarithmically growing P388 lymphoblasts were exposed for 15 min to a 5 μM concentration of the listed nucleosides. Present are the apparent concentrations of nucleosides (and/or its phosphorylated metabolites) interiorized by these cells based on the specific activity of the nucleoside used.

*ND, not determined.

Results are expressed as mean ±SD of eight MNNG-induced resistant cell lines.

---

**Table 3. Metabolism of ara-AC, deoxycytidine, and cytidine in P388 cells sensitive or resistant to ara-AC.**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Nucleoside</th>
<th>Monophosphate</th>
<th>Diphosphate</th>
<th>Triphosphate</th>
<th>Incorporation into DNA (pmol/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P388/S</td>
<td>ara-AC</td>
<td>0.8</td>
<td>1.0</td>
<td>3.4</td>
<td>&lt;0.25</td>
</tr>
<tr>
<td></td>
<td>Deoxycytidine</td>
<td>0.7</td>
<td>1.0</td>
<td>4.7</td>
<td>226</td>
</tr>
<tr>
<td></td>
<td>Cytidine</td>
<td>0.6</td>
<td>33.5</td>
<td>73.5</td>
<td>122</td>
</tr>
<tr>
<td>P388/RI-VIII*</td>
<td>ara-AC</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.25</td>
</tr>
<tr>
<td></td>
<td>Deoxycytidine</td>
<td>&lt;0.1</td>
<td>0.4 ± 0.2</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>Cytidine</td>
<td>0.6 ± 0.1</td>
<td>34.5 ± 8.4</td>
<td>69.5 ± 13.6</td>
<td>77.3</td>
</tr>
</tbody>
</table>

* Results are expressed as mean ±SD of eight MNNG-induced resistant cell lines.
**MECHANISMS OF RESISTANCE TO ARABINOSYL-5-AZACYTOSINE**

Table 4 *In vivo metabolism of ara-AC in P388 and colon 38 tumors*

<table>
<thead>
<tr>
<th>Tumor line</th>
<th>Drug dosage (mg/kg)</th>
<th>Drug or metabolite (nmol/g)</th>
<th>Incorporation into nucleic acids (nmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ara-AC</td>
<td>Monophosphate</td>
</tr>
<tr>
<td>P388</td>
<td>100</td>
<td>290 ± 32</td>
<td>18 ± 11</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>349 ± 235</td>
<td>144 ± 50</td>
</tr>
<tr>
<td>Colon 38</td>
<td>100</td>
<td>214 ± 68</td>
<td>7 ± 3</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>345 ± 106</td>
<td>78 ± 33</td>
</tr>
</tbody>
</table>

*° ara-ACXP, ara-AC converted to the nucleotide level. ND, not determined.

# Corrected by subtracting the amount of purine-associated radioactivity resulting from decomposition of ara-AC. (See Fig. 2.)

**Materials and Methods.**

[2,4-14C]ara-AC is not radioactive, so that the use of this isotopic form of the drug circumvents the problem of labeling of purines seen with [6-3H]ara-AC.

Enzyme Studies. The foregoing studies document a complete or partial enzymatic defect in the conversion of ara-AC to its phosphorylated forms in cells resistant to the drug. In order to determine the molecular basis for these metabolic results, the activities of relevant kinases and allied enzymes were determined in extracts of sensitive and resistant P388 lymphoblasts. The results of these analyses are presented in Table 5. Except for the activity of deoxycytidine kinase, the other purine and pyrimidine kinases examined showed no correlation with the nature of these cell lines, whether sensitive or resistant. The deaminases and phosphatases examined also did not vary with the acquisition of resistance. Deoxycytidine kinase, however, was found to be markedly (about 95%) depressed in all the resistant cell lines examined. These results thus provide a plausible explanation for the in vitro metabolic studies described earlier.

A limited examination of the relevant anabolic and catabolic enzymes was also conducted in colon 38 and P388 leukemia grown s.c. in mice. As listed in Table 6, extracts of colon 38 carcinoma exhibited a low activity of all the pyrimidine kinases examined: thus, the specific activity of deoxycytidine kinase was found to be 9-fold lower, thymidine kinase was 12-fold lower, uridine/cytidine kinase was 6-8-fold lower, and adenosine kinase was 6-fold lower than the respective specific activities observed in the s.c.-grown P388 tumor. It is also noteworthy that the nucleoside triphosphatase activity of colon 38 carcinoma (with ATP as substrate) was found to be 4-fold higher than in P388 tumors. This high triphosphatase activity could contribute to the resistance of colon 38 carcinoma to ara-AC, by serving to catabolize higher phosphorylated forms of the drug which are, presumably, the proximate antimitabolites.

Since deamination of ara-AC or ara-AC monophosphate would convert these molecules to uracil derivatives with low cytostatic potential, it appeared reasonable to determine whether the enzymes catalyzing these activities might also be associated with resistance to the drug in vivo. As shown in Table 6, both cytidine deaminase and deoxycytidyde deaminase exhibited higher specific activities in the resistant colon 38 carcinoma, when compared to the drug-sensitive P388 tumors. However, since cytidine deaminase does not attack ara-AC with effi-
MECHANISMS OF RESISTANCE TO ARABINOSYL-5’-AZACYTOSINE

Table 7  Nucleotide levels and incorporation of radiolabeled bicarbonate into the nucleoside triphosphates of P388 sensitive and resistant cell lines

<table>
<thead>
<tr>
<th>Nucleoside triphosphate</th>
<th>Sample</th>
<th>pCi incorporated/mmol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P388/S</td>
<td>P388/R I-VIII</td>
</tr>
<tr>
<td>ATP</td>
<td>2.22</td>
<td>2.46 ± 0.45</td>
</tr>
<tr>
<td>GTP</td>
<td>0.50</td>
<td>0.50 ± 0.10</td>
</tr>
<tr>
<td>CTP</td>
<td>0.23</td>
<td>0.36 ± 0.10</td>
</tr>
<tr>
<td>UTP</td>
<td>0.75</td>
<td>0.97 ± 0.25</td>
</tr>
</tbody>
</table>

* Endogenous nucleoside triphosphates were determined in acid-soluble cell extracts as described in "Materials and Methods.”
* Fifty ml of logarithmically growing cells (2–5 x 10⁶ cells/ml) were incubated with 100 µCi of [¹⁴C]bicarbonate for 30 min. After incubation the cells were centrifuged, and the pellet was extracted with 400 µl of 5% TCA. Neutralized supernatant was analyzed via HPLC. P388 sensitive and resistant cell lines (I through VIII) were assayed individually; mean values are shown for triplicate samples (deviation from mean, <±10%). Results for P388/R cell lines are represented as means ± SD of the eight cell lines assayed.
* ND, none detected.


cy, and since synthetic ara-AC-5’-monophosphate was not attacked by deoxycytidilate deaminase from colon 38, the relevance of the results with these enzymes to drug resistance is questionable.

Secondary Factors. In the interest of completeness, an evaluation of secondary factors, such as native nucleotide pools and the rates of their synthesis, was carried out in the P388 line and its resistant variants, after a brief exposure to [¹⁴C]bicarbonate. The results of these studies are presented in Table 7, where it can be seen that there are no significant differences in either the endogenous nucleoside triphosphates or in the rate of synthesis of pyrimidine triphosphates. This suggests that the mutagenic process used in the development of resistance to ara-AC did not alter the basal nucleotide pools or their synthetic rates. On the other hand, as shown in Fig. 2, the endogenous nucleoside triphosphate levels in the naturally resistant carcinoma colon 38 were significantly lower than the corresponding nucleotide levels in s.c.-grown P388, despite the fact that the total nucleotide pools were higher in colon 38. These results correlate well with the higher nucleoside triphosphatase activity observed in colon 38 carcinoma (Table 6). Such secondary factors, operating in conjunction with a relative kinase deficiency, provide a plausible pluricausal basis for the inherently resistant nature of colon 38 toward ara-AC.

Sensitivity to ara-AC in Human Tumor Cells. To determine whether these studies in murine tumor systems were applicable to human tumors, we examined a panel of six representative human malignant cell lines for their sensitivity to the drug and for their levels of deoxycytidine kinase and a number of other potentially relevant enzymes. As indicated in Fig. 3, the lowest IC₅₀ values of ara-AC were measured in the two lines with the highest deoxycytidine kinase activity (NCI-H358 and THP-5), whereas the highest IC₅₀ value was measured in the strains most deficient in this enzyme (late-passage NCI-H460). Between these extremes, the correlation was difficult to establish in a stringent way because of the small number of cell lines examined.

DISCUSSION

In the present paper, an attempt has been made to identify those factors which render tumors resistant to ara-AC. Three types of resistance were studied: natural resistance, exemplified by the colon 38 carcinoma, a transplantable neoplasm showing no therapeutic response to ara-AC; acquired resistance produced in vitro by cultivation in the presence of incremental concentrations of the drug; and acquired resistance produced by mutagenesis with MNNG followed by selection of mutants through cloning in the presence of the drug.

In all three cases, resistance was accompanied by a substantial reduction in the ability of the tumors or tumor cells to carry out the phosphorylation of ara-AC. This defect was ultimately attributable to a depression or deletion of deoxycytidine kinase in the refractory lines, an enzymatic lesion also seen in tumors resistant to the congeneric nucleoside ara-C (14, 15).

In addition to its comparative deficiency of deoxycytidine kinase, the colon 38 carcinoma was also endowed with an efficient nucleoside triphosphatase, the elevated activity of which was probably responsible for reducing the steady-state concentration of all the ribonucleoside triphosphates in this tumor (6) (Fig. 2). If, as seems possible, this enzyme was also to attack the triphosphate of ara-AC [these enzymes customarily exhibit broad specificity (16)], it would preclude the maintenance of effective levels of this proximate antimetabolite and thereby contribute in an important way to the resistant state. Although it might seem likely that the therapeutic activity of any nucleoside drug the activity of which depends on the generation and maintenance of adequate levels of its 5’-triphosphate would be compromised in this transplantable tumor, it is of interest that colon 38 carcinoma is sensitive to 5'-fluorouracil, 6-thioguanine and several lipophilic prodrugs of arabinosylcytosine, so that additional factors must be operative in these cases.

The enzymological studies with cultured human cells re-


* D. A. Cooney, unpublished observations.
MECHANISMS OF RESISTANCE TO ARABINOSYL-5-AZACYTOSINE

Fig. 3. A, inhibitory effects of ara-AC on the cloning of human lung tumor cells; B, inhibitory effects of ara-AC on the proliferation of human and murine tumor cells; C, correlation between deoxycytidine kinase activity and IC<sub>50</sub> value of ara-AC in the tumor lines depicted in A and B. Methods of measurement of cloning efficiency, growth rate and enzyme activities are described in the text.

ported in this paper were of limited scope inasmuch as only six types of tumor were included in the analyses. Nevertheless, it seems safe to predict that a tumor with a rich endowment of deoxycytidine kinase (exemplified in these experiments by the pulmonary carcinoma H358) will be outstandingly sensitive to the cytotoxic effects of ara-AC (cf. Fig. 3) on the grounds that such a tumor will effectively convert the drug to the proximate antitumor species (ara-AC-triphosphate) before major chemical decomposition of the molecule can occur.

Viewed overall, the present results warrant the expectation that measurements of deoxycytidine kinase in the tumors of patients scheduled to receive ara-AC during Phase I/II clinical trials might be of use in predicting their responsiveness to the drug. In the context of the behavior of the tumor cells examined in the present study, it seems probable that cells deficient in this enzyme will be refractory to the drug. Our results with the colon 38 carcinoma also make it likely that high levels of the nucleoside triphosphatase will confer resistance to ara-AC.

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Arabinosyl-5-azacytosine: Mechanisms of Native and Acquired Resistance


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