Differential Effect of N-(Phosphonacetyl)-L-aspartate on 1-ß-D-Arabinofuranosylcytosine Metabolism and Cytotoxicity in Human Leukemia and Normal Bone Marrow Progenitors

Steven Grant, Frank Rauscher, III, and Ed Cadman

ABSTRACT

The effect of N-[phosphonacetyl]-L-aspartate (PALA) on the metabolism and cytotoxicity of 1-ß-D-arabinofuranosylcytosine (ara-C) was studied in the human promyelocytic leukemic cell line, HL-60, and in normal human bone marrow. HL-60 cells exposed to 0.1 mM PALA for 12 hr accumulated 58.7 pmol ara-C per 10⁶ cells after a 45-min exposure to 1 µM ara-C, compared to 27.8 pmol ara-C per 10⁶ cells in untreated control cells. This PALA concentration and exposure interval was compared to untreated control HL-60 cells. Exposure of HL-60 cells to PALA followed by ara-C produced greater than additive effects on the inhibition of DNA synthesis, the inhibition of cell growth, and clonogenicity. In contrast, exposure of normal human bone marrow to the same PALA-ara-C schedule was not associated with a synergistic inhibition of colony-forming units in soft agar. If these perturbations also occur in vivo, an improvement in the therapeutic index of ara-C in patients with acute leukemia might result.

INTRODUCTION

ara-C⁴ is a deoxycytidine analog which is effective for the treatment of human acute myelogenous leukemia (5, 15). The mechanism of action of ara-C is controversial. ara-CTP is considered the lethal nucleotide form of ara-C, exerting its mechanism of action in DNA synthesis have resulted in an augmentation of ara-C metabolism and cytotoxicity following dThd treatment has been correlated with reductions in dCTP pool sizes (20). Similarly, 3-deazauridine, an inhibitor of CTP synthetase (38), produces reductions in intracellular dCTP levels (17) and enhanced ara-C activation (39). The study of these antimetabolite interactions forms the scientific foundation of ongoing clinical trials in humans involving the administration of PALA-ara-C schedule was not associated with a synergistic inhibition of colony-forming units in soft agar. If these perturbations also occur in vivo, an improvement in the therapeutic index of ara-C in patients with acute leukemia might result.

1 Supported by Grants CA-24187, CA-27130, and CA-08341 from the National Cancer Institute; Grants CH-145 and CA-5-61659 from the American Cancer Society; and the Celare Burtin Foundation for Cancer Research and the Zinberg Foundation for Research. Presented in part at the 72nd Annual Meeting of the American Association for Cancer Research, Washington, D. C., April 27 to 30, 1981 (19).
2 Recipient of the Pharmaceutical Manufacturers Research Starter Award. Fellow of the Leukemia Society of America. To whom requests for reprints should be addressed.
3 Recipient of a Faculty Research Award from the American Cancer Society.
4 The abbreviations used are: ara-C, 1-ß-D-arabinofuranosylcytosine; ara-CTP, 1-ß-D-arabinofuranosylcytosine-5'-triphosphate; dThd, thymidine; PALA, N-(phosphonacetyl)-L-aspartate; CFU-GM, colony-forming units of granulocyte and monocyte stem cells, RPMI 1640, Roswell Park Memorial Institute Tissue Culture Medium 1640; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; dNTP, deoxynucleoside triphosphate.
5 Received January 11, 1982; accepted June 28, 1982.
Nuclear Chicago, Chicago, Ill.). Intracellularly incorporated drug was the HCICx. The tubes were frozen, sectioned into separate compart-
ments, and placed in scintillation vials containing 10 ml of aqueous
99.99% of the free radiolabeled drug remained in the top layer, and
radioactivity was determined, using a Uniflux II scintillation counter.

Intracellular Drug Accumulation Studies

Briefly, 200 μl of the cell suspension were placed in a microfuge tube
and 10% heat-inactivated fetal calf serum (Grand Island Biological Co.,
Grand Island, N. Y.) at 37° and 5% CO₂. Cells were passaged twice
weekly and routinely screened for Mycoplasma contamination. All
experiments were performed utilizing cells in mid-logarithmic growth
(concentration, 2 to 4 x 10⁵ cells/ml).

Drugs and Chemicals

PALA (NSC-224131) was provided by Dr. Robert Engle, Develop-
mental Therapeutics Program, National Cancer Institute, Bethesda,
Md. It was stored as a dry powder at 0°, and reconstituted in sterile
0.9% NaCl solution prior to each use. ara-C and deoxycytidine were
purchased from Sigma Chemical Co., St. Louis, Mo. [5-3H]ara-C (26
Ci/mmol), [6-3H]dThd (22 Ci/mmol), and [5-3H]deoxycytidine (20 Ci/
mmol) were purchased from Amersham Radiochemicals, Arlington
Heights, Ill.

Intracellular Drug Accumulation Studies

HL-60 cells were placed in 25-cm² sterile tissue culture flasks
(Corning Glass Works, Corning, N. Y.) and were exposed to concen-
trations of PALA ranging from 0.01 to 1.0 mm. The flask were then
placed in a 37°, 5% CO₂ humidified incubator. At the end of the incu-
basation period, the cells were transferred to sterile, disposable 15-
ml centrifuge tubes, and spun at 1200 rpm for 8 min, the drug-
containing medium was discarded, and the cell pellet was washed
twice with fresh medium. The cells were then resuspended in 2 ml of
fresh RPMI 1640 with 20 μl of 1% HEPES buffer and placed in a
metabolic shaking water bath at 37°. To each flask was added 1 μM
ara-C, and the reaction flasks were placed in the shaking metabolic water
bath at 37°, and the cells were exposed to the appropriate concentra-
tion of ara-C in the presence of 5 μCi of [3H]dThd (22 Ci/mmol). At the
end of a 45-min incubation period, 750 μl of the cell suspension were
filtered through glass fiber filter paper (Whatman, Inc., Clifton, N. J.),
utilizing a 12-channel filtration manifold. The cells were lysed with cold
10% HClO₄, and washed 3 times with cold distilled water alternating
with cold 5% HClO₄. The filters were then air dried and placed in
scintillation vials containing 10 ml of nonaqueous scintillation fluid
(Betafluor, National Diagnostics), and the radioactivity was determined.
Inhibition of DNA synthesis, reflected by a decrease in [3H]dThd incor-
porated into the acid-precipitable HL-60 cell fractions relative to control
cells, is expressed as cpm/10⁶ cells. ara-C-induced inhibition of DNA
synthesis is compared for control and PALA-treated cells.

Chart 1. Interaction between ara-C metabolism and PALA inhibition of de
novο pyrimidine synthesis. AraCMP, 1-β-D-arabinofuranosylcytosine 5’-mono-
phosphate; AraCDP, 1-β-D-arabinofuranosylcytosine 5’-diphosphate.

ara-CTP Generation and Retention

The generation of ara-CTP in HL-60 cells following PALA pretreat-
ment was determined as we have described previously (20). After
PALA exposure, cells were washed twice with fresh medium, resus-
pended in 2 ml of RPMI 1640 containing 10% fetal calf serum and 20
μl 1% HEPES buffer, and placed in a 37° water bath. After a 45-min
exposure to 1 μM [3H]ara-C, the cells were centrifuged at 1200 rpm for
8 min at 4°. The cell pellet was washed twice in cold RPMI 1640 and
precipitated with 0.75 ml of cold 0.5 M HClO₄. After centrifugation at
1200 rpm for 8 min at 4°, the acid-soluble extract, containing the
ribonucleotides, was neutralized with cold 4 N KOH. After repeated
centrifugation and removal of the precipitated KClO₄, the acid-soluble
extracts were stored at -20° until all samples could be analyzed at
the same time. Separations were performed on an Altex Model 332
high-pressure liquid chromatographic system (Altex Instruments,
Berkeley, Calif.), utilizing a Partisil-10 SAX anion exchange resin (4.6
mm x 0.25 cm; Whatman, Inc.) and an isocratic 0.4 M Na₂HPO₄ buffer
(pH 3.3) at a flow rate of 1.5 ml/min. Absorbances were recorded at
254 and 280 nm, and 30-sec fractions were collected. The amount of
ara-CTP was determined by quantitating the amount of radioactivity
coeuluting with the nonradiolabeled ara-CTP standard. Significant quan-
tities of 1-β-D-arabinofuranosyluracil 5’-triphosphate were not noted
under conditions of this experiment.

For determination of ara-CTP retention, cells exposed sequentially

Effect of PALA on ara-C Metabolism

Deoxyribonucleotide Analysis

Alterations in intracellular dNTP concentrations in HL-60 cells following exposure to various PALA doses and exposure intervals were determined, utilizing a modification of the technique of Solter and Handschumacher (50). The details of this method have been described by us previously (20). Extracts of the HL-60 cells following PALA pretreatment were prepared according to the method of Lowe and Grindey (36). The standard reaction mixture contained the following in a final volume of 0.1 ml: 6 μmol glycine-NaOH buffer (pH 9.2); 0.5 μmol MgCl₂; 0.09 μmol 2-mercaptoethanol; 0.6 unit Escherichia coli DNA polymerase I (Worthington Biochemical Corp., Freehold, N. J.); 3 μg of nicked calf thymus DNA; and 600 pmol of each of the triphosphates to be used in excess, including the radiolabeled dNTP (specific activity, 300 μCi/mmol). The labeled dNTP used was [5-³H]dCTP, [5-³H]dGTP, [5-³H]dATP, or [5-³H]dTTP (American Radiolabeled Chemicals, Inc., St. Louis, Mo.). The plates were then incubated at 18° for 90 min. The reaction was terminated by placing the plates in an ice bath, and 80 μl of each well were then spotted on Whatman 3MM filter discs. These discs were then washed twice by placing them in 5% trichloroacetic acid:1% sodium pyrophosphate (10-ml filter) and washed once in 95% ethanol. The discs were then air dried and placed in minivials, 3 ml of Econofluor were added (New England Nuclear, Waltham, Mass.), and the radioactivity incorporated into newly synthesized DNA was determined.

RESULTS

Intracellular Accumulation of [³H]ara-C

The effect of various PALA concentrations and exposure intervals on the intracellular accumulation of ara-C in HL-60 cells is represented in Table 1. A 12-hr exposure to 0.1 mM PALA was associated with the intracellular accumulation of 58.7 pmol ara-C per 10⁶ cells, compared to 27.8 pmol ara-C per 10⁶ cells in untreated control cells. Lower PALA concentrations...

<table>
<thead>
<tr>
<th>PALA (mM)</th>
<th>Exposure (hr)</th>
<th>Intracellular ara-C (pmol/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>27.8 ± 4.3³</td>
</tr>
<tr>
<td>0.05</td>
<td>6</td>
<td>29.7 ± 3.4</td>
</tr>
<tr>
<td>0.05</td>
<td>12</td>
<td>34.8 ± 3.3</td>
</tr>
<tr>
<td>0.05</td>
<td>24</td>
<td>30.4 ± 3.8</td>
</tr>
<tr>
<td>0.1</td>
<td>6</td>
<td>36.9 ± 5.2</td>
</tr>
<tr>
<td>0.1</td>
<td>12</td>
<td>58.7 ± 5.4</td>
</tr>
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<td>0.1</td>
<td>24</td>
<td>48.7 ± 4.2</td>
</tr>
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<td>1.0</td>
<td>6</td>
<td>32.7 ± 3.8</td>
</tr>
<tr>
<td>1.0</td>
<td>12</td>
<td>42.1 ± 6.3</td>
</tr>
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Table 1: Total intracellular accumulation of [³H]ara-C by HL-60 cells following exposure to PALA

³ Mean ± S.D.

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</tr>
</thead>
<tbody>
<tr>
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<td>27.8 ± 4.3³</td>
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<td>6</td>
<td>29.7 ± 3.4</td>
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<tr>
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<td>12</td>
<td>34.8 ± 3.3</td>
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<tr>
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<td>24</td>
<td>30.4 ± 3.8</td>
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<tr>
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<td>6</td>
<td>36.9 ± 5.2</td>
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</tr>
<tr>
<td>1.0</td>
<td>24</td>
<td>34.2 ± 4.2</td>
</tr>
</tbody>
</table>

³ Mean ± S.D.
trations (0.05 mM) and shorter exposure intervals (6 hr) resulted in small increments in ara-C accumulation. Longer exposure intervals (24 hr) and higher PALA concentration (0.2 and 1.0 mM) also resulted in increments in intracellular ara-C accumulation which were generally less than those associated with a 12-hr exposure to 0.1 mM PALA.

DNA Inhibition

The effects of PALA on ara-C-induced DNA inhibition is shown in Chart 2. Exposure of HL-60 cells to 0.1 mM PALA for 12 hr and 50 nM ara-C for 45 min produced 8 and 24% decrements in the acid-precipitable incorporation of $[^3H]dThd$, respectively. Sequential exposure of cells to 0.1 mM PALA for 12 hr followed by 50 nM ara-C for 45 min resulted in an 88% inhibition of DNA synthesis. Because of the sharp dose-response curve of ara-C-induced DNA inhibition, the effects of PALA in conjunction with higher (≥100 nM) or lower (<25 nM) concentrations of ara-C were only additive (not shown).

ara-CTP Generation and Retention

The effect of PALA on the generation and retention of ara-CTP is illustrated in Table 2. Cells exposed to 0.1 mM PALA for 12 hr accumulated 36.9 ± 2.4 pmol ara-CTP per 10⁶ cells, compared to 16.4 ± 1.8 pmol ara-CTP per 10⁶ cells for untreated control cells. The corresponding 4-hr retentions of ara-CTP were 11.5 ± 1.6 and 4.6 ± 0.8 pmol/10⁶ cells, respectively. As with ara-C intracellular accumulation, lower (0.5 mM) and higher (1 mM) PALA concentration, as well as longer incubation intervals (24 hr), produced smaller increments in ara-CTP formation and retention.

Cloning Studies

HL-60 Cells. The effects of prior exposure to PALA on the soft-agar clonogenicity of HL-60 cells in the continuous presence of ara-C is shown in Table 3. A 12-hr exposure to 0.1 mM PALA resulted in an 8% reduction in colony relative to untreated control cells. The continuous presence of 10 nM ara-C was associated with a 24% reduction in cloning efficiency. The sequential exposure of HL-60 to 0.1 mM PALA for 12 hr, followed by plating in the presence of 10 nM ara-C, resulted in an 86% reduction in colony formation. Prior exposure to PALA in conjunction with higher concentrations of ara-C (e.g., 20 nM) produced a greater inhibition of cloning efficiency, but the degree of drug synergism was reduced (not shown).

Human Bone Marrow Cells. Table 4 illustrates the effect of pretreatment with PALA on ara-C-induced inhibition of colony formation of normal granulocyte-monocyte bone marrow progenitor cells (CFU-GM) in soft agar. In 8 separate experiments, exposure of cells to 0.1 mM PALA for 12 hr resulted in 73.1 ± 17.4% of control colony formation, whereas a continuous exposure to 10 nM ara-C resulted in 47.9 ± 12.9% of control cells. In cells exposed to 0.1 mM PALA for 12 hr, followed by continuous exposure to 10 nM ara-C, colony formation was reduced to 35.5 ± 14.2%. This sequence was only additive.

<table>
<thead>
<tr>
<th>PALA (mM)</th>
<th>Exposure (hr)</th>
<th>ara-CTP generation (pmol/10⁶ cells)</th>
<th>ara-CTP retention (pmol/10⁶ cells)</th>
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<tr>
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<td>24</td>
<td>16.8 ± 2.3</td>
<td>5.2 ± 1.2</td>
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a Mean ± S.D.

<table>
<thead>
<tr>
<th>Condition</th>
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<tr>
<td>Control</td>
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<tr>
<td>PALA (0.1 mM), 12 hr</td>
<td>92 ± 5</td>
</tr>
<tr>
<td>ara-C (10 nM), continuous</td>
<td>78 ± 6</td>
</tr>
<tr>
<td>PALA (0.1 mM), 12 hr before adding ara-C (10 nM)</td>
<td>14 ± 3</td>
</tr>
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</table>

a Mean ± S.D.
Effect of PALA on ara-C Metabolism

Table 5

<table>
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<tr>
<th>Exposure (hr)</th>
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<th>(pmol/10⁶ cells)</th>
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<td>4.7 ± 5</td>
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* Mean ± S.D.
* UD, undetectable.

for inhibition of bone marrow colony formation (73.1 x 47.9 = 35.0). These results contrast sharply to those obtained in HL-60 cells, where similar PALA and ara-C doses and exposure intervals resulted in greater than additive inhibitory effects. A 24-hr exposure of normal bone marrow cells to 0.1 mM PALA, followed by continuous exposure to 10 mM ara-C, also resulted in only additive effects on colony formation. In other experiments utilizing higher concentrations of ara-C (e.g., ≥20 mM), PALA either had no or little effect on further reduction in cloning efficiency of normal bone marrow progenitors. However, the substantial inhibition of clonogenicity associated with these higher ara-C concentrations (≥96%) makes interpretation of these results difficult.

Deoxynucleotide Levels

Alterations in dNTPs in HL-60 cells following PALA exposure are illustrated in Table 5. A 12- or 24-hr exposure to PALA concentrations of 0.5 mM or higher reduced dCTP pools below or to barely detectable levels (less than 0.5 pmol/10⁶ cells). A 0.1 mM PALA concentration also reduced intracellular levels of dCTP and dTTP but not to the extent of the higher PALA concentrations. Shorter PALA exposure intervals (e.g., 6 hr) were associated with small decreases in intracellular dCTP levels (not shown).

DISCUSSION

Efforts to improve the efficacy of nucleoside analogs such as ara-C by combining them with inhibitors of de novo pyrimidine synthesis have a rational biochemical basis. Early studies by Potter (46) have shown that combinations of agents which block separate steps in a linear metabolic pathway are likely to produce synergistic tumoricidal effects. If, in addition, one such agent were capable of enhancing the metabolism of another, further antitumor activity might be expected to occur. Previous work has suggested that diverse agents, such as dThd (12, 20, 31, 44), hydroxyurea (44, 55), 3-deazauridine (7, 44), pyrazofurin (44), and methotrexate (10), are capable of enhancing ara-C metabolism in murine tumor cells, in some cases resulting in synergistic antitumor effects. The common mechanism underlying these interactions is believed to be the depletion of intracellular levels of dCTP, the naturally occurring nucleotide which antagonizes the activation of ara-C by inhibiting deoxycytidine kinase (26). Based on considerations such as these, clinical trials involving the sequential administration of 3-deazauridine or dThd in conjunction with ara-C have been designed for patients with refractory acute nonlymphocytic leukemia (1, 4).

PALA, a transition state inhibitor of aspartate transcarbamylase, is similarly capable of enhancing ara-C metabolism and cytotoxicity in a human promyelocytic cell line. In contrast to agents such as dThd, hydroxyurea, or dihydro-2H-pyrazolo imidazole, which are inhibitors of ribonucleotide reductase, PALA inhibits at a considerably more proximal step in de novo pyrimidine synthesis. Exposure of HL-60 cells to 0.1 mM PALA for 12 or 24 hr produced a decrease in intracellular dCTP levels, a result which is comparable to that seen in L1210 cells following dThd exposure (12, 20, 31). This decrease was associated with an increase in the total intracellular accumulation of ara-C, as well as the generation and retention of the lethal ara-C metabolite, ara-CTP. The latter perturbation may be of particular significance, since recent clinical studies in patients with acute myelogenous leukemia have shown that leukemic cell retention of ara-CTP may correlate closely with response to this agent (48). Another possible determinant of ara-C cytotoxicity is its incorporation into nucleic acids, specifically DNA, with resultant inhibition of chain elongation or the formation of abnormal DNA (35). It is likely that PALA-induced enhancement of ara-C metabolism would be associated with an increase in DNA incorporation of the analog, and this might in part account for the increased cytotoxicity of these agents when administered in sequence. PALA-induced perturbations in ara-C metabolism are reflected biologically by enhanced inhibition of DNA synthesis and clonogenicity in soft agar. These effects are most pronounced at low (e.g., ≤50 mM) ara-C concentrations and may represent enhancement of ara-C metabolism within a kinetically susceptible subgroup of cells. Resistance of leukemic cells to ara-C may exist on either a biochemical (reduced formation of ara-C metabolites) or on a kinetic basis (decreased percentage of G1-S-phase cells) (47). Increased ara-C activation following PALA administration may in part overcome resistance secondary to biochemical factors but would not be expected to increase susceptibility in the kinetically resistant subgroup of cells.

The time course of optimal drug scheduling in HL-60 cells differs from that observed in the rapidly dividing murine leukemia L1210. Whereas a short (e.g., 3 to 6 hr) antimetabolite incubation period may produce maximal biochemical alterations in the murine line (20), a considerably longer exposure interval (e.g., 12 to 24 hr) may be required in human leukemic cells, whose doubling time is approximately 36 hr (32). The depletion of intracellular dCTP may depend upon multiple factors, including degree of inhibition of de novo synthetic pathways, availability of salvage pyrimidines, activity of catalytic enzymes such as deoxycytidine deaminase, and rate of utilization of nucleotides in DNA synthesis (37). In slowly replicating human leukemia cells such as HL-60, longer PALA exposure intervals may be required to allow sufficient numbers of cells to pass through the DNA-synthetic phase, resulting in depletion of available dCTP. These scheduling considerations may have complications for the design of in vivo trials involving antimetabolites such as PALA.

The ability of one antimetabolite to enhance the metabolism...
of another will not result in a net increase in therapeutic index, unless these alterations occur selectively in tumor cells. A recent review by Preisler (47), examining the ability to predict a response in leukemia, indicates our current inability to selectively kill leukemic cells. To date, single agents have shown similar patterns of toxicity with respect to inhibition of clonogenicity of leukemic versus normal bone marrow progenitors (2, 9, 14, 25, 33, 34, 43, 45, 52). Our study shows that a particular PALA dose and schedule is capable of selectively enhancing the in vitro cytotoxicity of ara-C in a human promyelocytic cell relative to normal bone marrow precursors. In early clinical trials, PALA has demonstrated little myelosuppression (23). It is possible that elevated levels of aspartate transcarbamylase in myeloid progenitors may protect the cells from the action of PALA, although the inability to obtain sufficient numbers of bone marrow stem cells to perform enzymatic assays makes this hypothesis difficult to confirm. Whether these in vitro results will produce improved selectivity of ara-C in vivo will depend on multiple factors, including the ability to achieve adequate and sustained plasma levels of PALA, the development of leukemic cells resistant to PALA, the response of normal bone marrow progenitors to PALA in an in vivo setting, and cytokinetic considerations, which may in some instances antagonize biochemical effects. Nevertheless, selective enhancement of ara-C by PALA in leukemic cells would appear to be a theoretical possibility, and in vivo trials to determine optimal schedules of these agents in sequence appear warranted.

REFERENCES


Effect of PALA on ara-C Metabolism


Differential Effect of \(N\)-(Phosphonacetyl)-l-aspartate on 1-\(\beta\) -d-Arabinofuranosylcytosine Metabolism and Cytotoxicity in Human Leukemia and Normal Bone Marrow Progenitors

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