Kinetic Studies on Phosphorylation of 5-Azacytidine with the Purified Uridine-Cytidine Kinase from Calf Thymus

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SUMMARY

Uridine-cytidine kinase was purified 330-fold from calf thymus. This enzyme catalyzes the phosphorylation of uridine, cytidine (CR), and the nucleoside analog, 5-azacytidine (5-aza-C) to their respective nucleoside 5'-monophosphates in the presence of Mg++ and adenosine 5'-triphosphate. The K_m values for CR uridine, and 5-aza-C were 40, 50, and 200 ¿M, respectively. Uridine 5'-triphosphate or cytidine 5'-triphosphate inhibited the phosphorylation of these nucleosides; the inhibition was noncompetitive with respect to the nucleoside substrates and competitive with respect to adenosine 5'-triphosphate. CR and uridine were potent competitive inhibitors of 5-aza-C (K_i values of 40 and 50 ¿M, respectively), whereas 5-aza-C was a weak competitive inhibitor of CR (K_i value of 200 ¿M).

INTRODUCTION

5-aza-C, a cytidine analog, is an antitumor agent active against acute leukemia (8, 15). The drug must be phosphorylated for activity presumably by uridine-cytidine kinase since both uridine and cytidine can antagonize the inhibitory effects of the analog (4, 7, 12). The cytotoxic activity of 5-aza-C results from the incorporation of the phosphorylated intermediates of this drug into nucleic acid (7, 18, 21) and probably to a lesser extent its inhibition of de novo pyrimidine biosynthesis (3).

Uridine-cytidine kinase catalyzes the phosphorylation of uridine or cytidine, in the presence of ATP and Mg++, to UMP and CMP. This enzyme, which is a part of the salvage pathway for pyrimidine ribonucleosides, has been isolated from rat liver (2), from ascites tumor cells (11, 17, 19, 20), and from sensitive and 5-aza-C-resistant mouse leukemia cells (25, 26). The activity of uridine-cytidine kinase increases during the S phase of the cell cycle (22). Both UTP and CTP are potent feedback inhibitors of this enzyme (1).

We have purified uridine-cytidine kinase from calf thymus and studied the phosphorylation of 5-aza-C. We have found that uridine-cytidine kinase catalyzes the phosphorylation of 5-aza-C and that UTP and CTP are potent inhibitors of the phosphorylation of this nucleoside analog.

MATERIALS AND METHODS

Uridine-Cytidine Kinase Assay. The enzyme assay measures the conversion of nucleoside-³H to nucleotide-³H by a differential binding of these latter compounds to DEAE-cellulose disc (5). The reaction mixture (0.1 ml) contained 5 µmoles of Tris-HCl, pH 7.5; 0.25 µmole of MgCl_2; 1 µmole of 2-mercaptoethanol; 0.2 µmole of ATP; 10 µmoles of cytidine-³H (3.3 to 5.5 X 10^5 cpm); and 0.2 to 2 units of enzyme. After the mixture was incubated at 37° for 10 min, the reaction was terminated by adding 5 ml of ice water. The solution was permitted to flow by gravity through 2.5-cm-diameter DEAE-cellulose discs that had been washed previously with 1 ml of 0.01 N HCl and 5 ml of water. The discs were then washed with 30 ml of water, dried, and counted in a scintillation fluid (10 ml) containing 5 g of PPO and 100 mg of POPOP in 1 liter of toluene. The vials were counted in a Packard Tri-Carb scintillation counter with a counting efficiency for tritium under these conditions of about 3%. One unit of enzyme activity was defined as the amount of enzyme catalyzing the formation of 1 nmole of CMP from cytidine per 10 min under these assay conditions.

Materials. The tritium-labeled purine and pyrimidine nucleosides were obtained from Schwarz/Mann, Orangeburg, N. Y. Nonradioactive nucleosides and nucleotides were obtained from Calbiochem, San Diego, Calif., and P-L Laboratories, Milwaukee, Wis. DEAE-cellulose powder (exchange capacity, 0.75 mEq/g), ammonium sulfate (enzyme grade), protamine sulfate, and Sepharose 6B were obtained from BioRad Laboratories, Richmond, Calif.; Schwarz/Mann; Sigma Chemical Co., St. Louis, Mo.; and Pharmacia Fine Chemicals, Piscataway, N. J., respectively. Calf thymus was obtained from a local abattoir; it was placed on ice after removal from the animal and stored at -20°.

Both 5-aza-C-¹⁴C and 5-aza-C (NSC 102816), supplied through the Chemical and Drug Procurement Section, Chemotherapy, National Cancer Institute, Bethesda, Md., were filtered through DEAE-cellulose discs immediately prior to their use for the kinetic study of 5-aza-C phosphorylation. CTP and UTP were purified by a column chromatography of DEAE-cellulose. The nucleotides were eluted by a gradient buffer in a linear increase of triethylammonium bicarbonate from 0 to 0.2 M. The nucleoside triphosphate was then concentrated in a rotary evaporator at room temperature. The purity of the nucleotides was checked by thin-layer chromatography on DEAE-cellulose-coated glass plates with 0.05 N HCl as eluting solvent.
RESULTS

Purification of Enzyme

Supernatant. Frozen calf thymus (606 g) was broken up into small pieces with a hammer and ice pick and suspended into 1000 ml of 50 mM Tris-HCl, pH 7.5; homogenized in a heavy duty commercial Waring Blender for 3 min; and centrifuged at 20,000 X g for 30 min. The supernatant (Fraction 1) was collected after passing through 4 layers of gauze. To the supernatant 0.9 ml of 14 M 2-mercaptoethanol was added. Throughout the purification procedure the temperature was maintained at about 4°.

Streptomycin Fractionation. To 965 ml of Fraction 1, 48.7 ml of 10% streptomycin sulfate, pH 7.0, were slowly added over a 10-min period with stirring. After being stirred for an additional 15 min, the suspension was centrifuged at 20,000 X g for 15 min to collect the precipitate (Table 1, Fraction 2). The enzyme activity was found in the precipitate.

Protamine Sulfate Fractionation. To 965 ml of Fraction 2, 195 ml of 2% protamine sulfate, pH 7.0, were added over a 10-min period with stirring. After being stirred for an additional 15 min, the suspension was centrifuged at 20,000 X g for 15 min. The supernatant was discarded. To the protamine precipitate, 200 ml of 2% ammonium sulfate containing 50 mM Tris-HCl, pH 7.5, and 20 mM 2-mercaptoethanol were added. The suspension was stirred for 60 min until homogenous and then centrifuged at 20,000 X g for 10 min. The supernatant was collected as Fraction 3.

Ammonium Sulfate Fractionation. To 200 ml of Fraction 3, 31 g of ammonium sulfate (0 to 26% saturation) were added with stirring over a 15-min period. The mixture was stirred for an additional 15 min and centrifuged at 30,000 X g for 10 min. The precipitate was discarded, and 11.9 g of ammonium sulfate (26 to 36% saturation) were added to 200 ml of the supernatant over a 10-min period. After an additional 15 min stirring, the precipitate was dissolved in 20 ml of 100 mM Tris-HCl, pH 7.5, containing 10 mM 2-mercaptoethanol (Fraction 4). The enzyme preparation could be stored frozen (−20°) at this stage with very little loss of activity.

Chromatography of Sepharose 6B. Fraction 4 was applied to a column of Sepharose 6B (19.4 sq cm x 90 cm), that had been previously equilibrated with 50 mM Tris-HCl, pH 7.5, and 20 mM 2-mercaptoethanol in 20% glycerol. The column was eluted with the same buffer by upward flow at a rate of 30 ml/hr. Fractions (7.5 ml) were collected and analyzed for protein and uridine-cytidine kinase activity (Chart 1). The fractions (65 through 71) of maximal activity and containing 43% of the activity applied to the column were pooled and dialyzed against the same buffer (Fraction 5).

Chromatography on DEAE-cellulose. Fraction 5 (50 mg of protein and 1880 units of enzyme in 40 ml of buffer containing 50 mM Tris-HCl, pH 7.5, and 20 mM 2-mercaptoethanol in 20% glycerol) was added to the top of a DEAE-cellulose column (1 sq cm x 8 cm) and allowed to drain by gravity. The column had previously been equilibrated with the same buffer. The chromatogram was developed by stepwise elution with KCl of increasing ionic strength. Fractions of 3.9 ml were collected and analyzed for absorbance at 280 nm and for enzyme activity. The active enzyme fraction was eluted with 100 mM KCl (Chart 2) and collected as Fraction 6. There was no loss of enzyme activity of Fraction 6 over a 3-month period when stored at −20°.

A summary of the purification procedure is given in Table 1. The specific activity of Fraction 6 was found to be about 330 times of that of the starting supernatant (Fraction 1).

Properties of Purified Enzyme

Properties of Reaction. The rate of formation of CMP from cytidine using ATP as the phosphate donor is linear with respect to enzyme concentration up to 10 min. Freeze-thaw of Fraction

Charts 1. Sepharose 6B chromatography of Fraction 4. Fraction 4 (21 ml) containing 813 mg of protein and 4370 units of enzyme activity was eluted from a column of Sepharose 6B (19.4 sq cm x 90 cm) with 50 mM Tris-HCl, pH 7.5; 20 mM 2-mercaptoethanol; and 20% glycerol. Fractions containing 7.5 ml were collected at 15-min intervals, and the uridine-cytidine kinase assay was performed as described under “Materials and Methods.”

Table 1

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Total units</th>
<th>Specific activity (units/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Extract</td>
<td>965</td>
<td>53,654</td>
<td>12,060</td>
<td>0.2</td>
</tr>
<tr>
<td>2. Streptomycin</td>
<td>965</td>
<td>34,161</td>
<td>11,380</td>
<td>0.3</td>
</tr>
<tr>
<td>3. Protamine</td>
<td>200</td>
<td>29,260</td>
<td>9,420</td>
<td>0.4</td>
</tr>
<tr>
<td>4. Ammonium sulfate</td>
<td>21</td>
<td>813</td>
<td>4,370</td>
<td>5.4</td>
</tr>
<tr>
<td>5. Sepharose 6B</td>
<td>40</td>
<td>50</td>
<td>1,880</td>
<td>37.7</td>
</tr>
<tr>
<td>6. DEAE-cellulose</td>
<td>3.9</td>
<td>10.5</td>
<td>798</td>
<td>76.0</td>
</tr>
</tbody>
</table>
6 for 5 times did not result in the loss of enzyme activity. The reaction had an absolute dependence on the presence of Mg**-.

The velocity of the reaction was maximal at about 2.5 mM Mg**. Further increase in Mg** ion concentration appeared to inhibit the kinase activity slightly (data not shown). This finding is similar to that of the uridine kinase from Ehrlich ascites tumor which is also inhibited by a high concentration of Mg** (20). The optimal Mg** ion concentration for the latter kinase (25 mM) was found to be 10-fold greater.

Identification of Product. Cytidine-3H was incubated in the reaction mixture as described under "Materials and Methods" with Fraction 6 as the enzyme. At the end of the incubation, the 3H-labeled nucleoside monophosphates were isolated by thin-layer chromatography on DEAE-cellulose (6) with 0.01 N HCl as the solvent. The nucleotide-3H were eluted from the DEAE-cellulose with 0.5 M triethylammonium bicarbonate, pH 8.0, and recovered after evaporation of the eluting solvent. Chromatography of CMP-3H on microcrystalline cellulose (Avicel) with the use of isopropyl alcohol: NH₄OH:H₂O (60:30:10) as the solvent gave an Rf identical with 5'-CMP. Treatment of CMP-3H with Crotalus adamanteus 5'-nucleotidase resulted in the removal of the phosphate group. No degradation of the reaction mixture was evident on both sides of the pH range.

Effect of pH on Rate of Reaction. Under conditions of the routine assay, the purified enzyme (Fraction 6) had a hyperbolic curve of response of reaction rate to pH. The optimum pH range was between 7.0 and 8.0 in the Tris-N₂-hydroxyethyl piperazine N'-2-ethanesulfonic acid buffer (data not shown). A significant reduction of the reaction rate was evident on both sides of the pH range. A similar pH optimum range was also observed for the kinase from Novikoff ascites tumor (10) and Ehrlich ascites tumor (20), although the enzyme from Ehrlich ascites tumor exhibited its maximal velocity over a much broader pH range (5.5 to 8.0).

Specificity for Phosphate Acceptor. Fraction 6 was assayed for its ability to catalyze the phosphorylation of different nucleosides with ATP as the phosphate donor (Table 2). Cytidine and uridine were found to be the most active phosphate acceptors. The fact that none of the other nucleosides or deoxynucleosides could be used as phosphate acceptor indicated very little contamination of Fraction 6 with other kinases. 5-aza-C was found to be much less active as a phosphate acceptor than cytidine. Cytosine arabinoside was not phosphorylated at all.

Specificity for Phosphate Donor. Different nucleoside triphosphates were tested as phosphate donors with cytidine as the phosphate acceptor (Table 3). The data indicated that GTP, dATP, and dGTP could be used as phosphate donors for the phosphorylation of cytidine but are less active than ATP. dTTP is a poor phosphate donor for the cytidine kinase reaction. CTP, UTP, and dCTP showed no activity as phosphate donors.

Effect of Nucleosides and Deoxynucleosides on Phosphorylation of Cytidine. The amount of CMP-3H formed from cytidine-3H was not affected by the addition of either cold nucleosides or deoxynucleosides in 10-fold excess except for uridine. The latter nucleoside inhibited the conversion of cytidine-3H to CMP-3H by 75%. Since uridine is a natural substrate of this enzyme (see below), the increased conversion of cytidine to CMP in the presence of uridine is expected. On the other hand, the presence of cytosine arabinoside did not

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**Table 2**

<table>
<thead>
<tr>
<th>Phosphate acceptor</th>
<th>Nucleotide formed (nmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytidine</td>
<td>0.60</td>
</tr>
<tr>
<td>Uridine</td>
<td>0.96</td>
</tr>
<tr>
<td>5-aza-C</td>
<td>0.25</td>
</tr>
</tbody>
</table>

*Other nucleosides (adenosine, guanosine, deoxyadenosine, deoxyguanosine, deoxycytidine, deoxythymidine, and cytosine arabinoside) tested for being the phosphate acceptor yielded less than 0.01 nmole of the corresponding nucleotide in the same assay system.

**Table 3**

<table>
<thead>
<tr>
<th>Phosphate donor</th>
<th>CMP formed (nmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>1.25</td>
</tr>
<tr>
<td>GTP</td>
<td>0.89</td>
</tr>
<tr>
<td>CTP</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>UTP</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>dATP</td>
<td>0.67</td>
</tr>
<tr>
<td>dGTP</td>
<td>0.55</td>
</tr>
<tr>
<td>dCTP</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>dTTP</td>
<td>0.23</td>
</tr>
</tbody>
</table>
significantly affect the phosphorylation of cytidine.

Relative Kinase Activity towards Uridine, Cytidine, and Deoxyadenosine. The phosphorylation of uridine, cytidine, and deoxyadenosine was compared with the crude extract (Fraction 1), the ammonium sulfate fraction (Fraction 4), and the purified enzyme (Fraction 6) (Table 4). The kinase activity towards uridine was observed to be 60% greater than cytidine with both types of enzyme preparations.

Deoxyadenosine kinase activity (10) which was present to a significant amount in the crude extract was essentially absent from the purified enzyme. The removal of deoxyadenosine kinase activity during the purification of cytidine kinase suggested that there are 2 distinctive enzyme species.

Inhibition by CTP and UTP. The amount of inhibition produced by different concentrations of CTP and UTP was measured with either cytidine, uridine, or 5-aza-C as the phosphate acceptor and ATP as the phosphate donor (Chart 3). CTP appeared to be more effective than UTP as an inhibitor of phosphorylation by the kinase.

CTP and UTP at concentrations of 40 and 60 μM,

Table 4

<table>
<thead>
<tr>
<th>Enzyme fraction</th>
<th>UMP formed (nmoles)</th>
<th>CMP formed (n mole)</th>
<th>UMP:CMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Crude extract</td>
<td>0.11</td>
<td>0.06</td>
<td>1.83</td>
</tr>
<tr>
<td>4. Ammonium sulfate</td>
<td>1.08</td>
<td>0.66</td>
<td>1.64</td>
</tr>
<tr>
<td>6. DEAE-cellulose</td>
<td>0.96</td>
<td>0.60</td>
<td>1.60</td>
</tr>
</tbody>
</table>

Effect of CTP and UTP on Phosphate Acceptor. The effect of CTP or UTP on the reaction rate in the presence of different concentrations of cytidine is shown in Chart 4. The data in both cases have been plotted according to the method of Lineweaver and Burk (13). The inhibition produced in both plots appeared to be noncompetitive with the phosphate acceptor. The K_{m} and V_{max} values for cytidine were 40 μM and 1.05 nmoles of CMP per 5 min per 2.7 μg of protein, respectively, and the K_{m} and V_{max} values for uridine were 50 μM and 1.34 nmoles of UMP per 5 min per 2.7 μg of protein, respectively. As for the K_{m} of uridine of this enzyme, it is very close to 50 to 500 μM as reported for the kinase from Ehrlich ascites tumor (11, 20) but much lower than 270 μM determined for uridine-cytidine kinase of Novikoff ascites tumor (17). The only published figure on K_{m} for cytidine was 23 μM from Ehrlich ascites tumor kinase (20), which is lower than that of calf thymus kinase.

Effect of CTP and UTP on Phosphate Donor. The effect of CTP or UTP on the reaction rate in the presence of different concentrations of ATP with cytidine as phosphate acceptor is shown in Chart 5. The inhibition produced by CTP or UTP appeared to be competitive with ATP. The K_{m} and V_{max} values of ATP determined from the Lineweaver-Burk plot were 140 μM and 1.43 nmoles of CMP per 5 min per 2.7 μg of protein, respectively; the K_{m} for this enzyme appeared to be lower than that of Novikoff ascites tumor kinase (310 μM). As to the K_{m} values of ATP for uridine kinase from Ehrlich ascites tumor, 2 values, 50 and 500 μM, were reported (11, 20).

Inhibition by Uridine or 5-aza-C. The effect of uridine or 5-aza-C on the rate of formation of CMP-3H with different concentrations of cytidine-3H is shown in the Lineweaver-Burk plots of Chart 6. The presence of either uridine or 5-aza-C produces a competitive inhibition of the phosphorylation of cytidine-3H. The K_{i} for uridine and 5-aza-C under these conditions were 53 and 200 μM, respectively.

Inhibition of 5-aza-C Phosphorylation by Cytidine and Uridine. The effect of cytidine or uridine on the rate of

K_{i} (S-5'U uridine)
Chart 5. Effect of ATP concentration on the inhibition produced by CTP and UTP. The reaction mixture (0.1 ml) contained 5 μmoles of Tris-HCl, pH 7.5; 0.25 μmole of MgCl₂; 1 μmole of 2-mercaptoethanol; 0.2 μmole of ATP; 2 units of Fraction 6; 10 nmoles of cytidine-³H, 5.5 x 10⁴ cpm; and the indicated concentrations of ATP and CTP or UTP. The mixture was incubated at 37° for 5 min and assayed as described under “Materials and Methods.”

Formation of 5-aza-CMP-¹⁴C using different concentrations of 5-aza-C-¹⁴C is shown in Chart 7. The Kₘ and Vₘₐₓ values for 5-aza-C determined from the Lineweaver-Burk plot were 200 μM and 0.32 n mole of 5-aza-CMP per 6 min per 2.7 μg of protein. The presence of cytidine and uridine in the reaction mixture produces an inhibition of the phosphorylation of 5-aza-C-¹⁴C, which appeared to be competitive with respect to this analog. The Kᵢ values for cytidine and uridine under these conditions were estimated to be 40 and 54 μM, respectively.

DISCUSSION

Uridine-cytidine kinase was purified 330-fold from calf thymus by ammonium sulfate and protamine fractionation and by column chromatography on Sepharose 6B and DEAE-cellulose (Table 1). Among the various nucleosides tested as substrates, this enzyme catalyzed the phosphorylation only of uridine, cytidine, and 5-aza-C (Table 2). All the naturally occurring nucleoside 5'-triphosphates could serve as a phosphate donor for uridine-cytidine kinase with the exception of UTP, CTP, and dCTP (Table 3). The estimated molecular weight of this enzyme is about 60,000 as determined by its elution volume from a column of Sepharose 6B (Chart 1).

Both UTP and CTP are potent feedback inhibitors of uridine-cytidine kinase (Chart 3). The inhibition produced by these nucleotides appears to be competitive with respect to ATP (Chart 5) and noncompetitive with respect to cytidine (Chart 4). The inhibition of the phosphorylation of 5-aza-C by UTP or CTP is slightly greater than the inhibition of the phosphorylation of uridine or cytidine by these nucleotides.
(Chart 3). A concentration of about 40 μM UTP or CTP inhibits the phosphorylation of 5-aza-C by more than 50%, the inhibition produced by CTP being greater than that produced by UTP.

In kinetic studies the K_m values for cytidine, uridine, and 5-aza-C were estimated to be 40, 50, and 200 μM, respectively (Charts 4, 5, and 7). The presence of a symmetrical triazole ring in 5-aza-C apparently reduces the binding affinity of this analog for the catalytic site of uridine-cytidine kinase. In addition the enzyme has more difficulty in catalyzing the transfer of a phosphate group from ATP to 5-aza-C than to cytidine since the V_max values for 5-aza-C is one-third the V_max value for cytidine.

Since uridine, cytidine, and 5-aza-C compete for the same catalytic site on uridine-cytidine kinase, these nucleosides are competitive inhibitors of each other. Cytidine and uridine are weak competitive inhibitors of the phosphorylation of 5-aza-C (Chart 7), the K_i values of uridine and cytidine being 40 and 50 μM, respectively. On the other hand, 5-aza-C is a weak competitive inhibitor of the phosphorylation of cytidine (Chart 5), the K_i value for this analog being about 200 μM.

The ability of uridine or cytidine to antagonize the growth inhibition produced by 5-aza-C (4, 7, 12) and the incorporation of radioactive 5-aza-C into nucleic acids of mammalian cells (12) suggest that the active form of 5-aza-C is a nucleotide. It appears that the rate-limiting step in the conversion of 5-aza-C to its respective nucleotides is the phosphorylation by uridine-cytidine kinase since the activity of the nucleoside transport system (9), CMP kinase (23), and nucleoside diphosphokinase (16) are comparably high in mammalian cells. Since uridine-cytidine kinase activity increases during the S phase of the cell cycle, this may be in part responsible for the greater cytotoxicity produced by 5-aza-C on S-phase cells than on cells in other phases (22). Another aspect of the increased cytotoxicity produced by 5-aza-C in S-phase cells may be due to the incorporation of this fraudulent nucleoside into DNA.

A concentration of 5-aza-C of 1 μg/ml (or 4 μM) for 2 hr is able to produce a greater than 70% kill of L1210 leukemic cells in vitro (12). Since this concentration of 5-aza-C is well below its K_m value of 200 μM for uridine-cytidine kinase and since the intracellular concentration of UTP and CTP is about 100 μM (24, 27), a level that should produce a potent inhibition of this enzyme, the amount of 5-aza-C nucleotide formed in the L1210 cells under these conditions must be very small. Since these low concentrations of 5-aza-C do not inhibit RNA or DNA synthesis (12), it is possible that the major factor responsible for cytotoxicity is the incorporation of 5-aza-C into RNA and DNA.

5-aza-C can produce complete remissions in acute leukemia when given at a dose of 150 mg/sq m by rapid i.v. injection daily for 5 days (8). Assuming a uniform distribution of 5-aza-C in the body fluids, this dose would give an estimated initial plasma level of approximately 5 μg/ml or 20 μM. Since 5-aza-C undergoes spontaneous chemical decomposition to presumably inactive products with a half-life of about 30 min (14), the estimated plasma levels of this antimetabolite for a 2-hr interval after i.v. injection would be about 1 to 20 μM. This level should be significantly cytotoxic to the human leukemic cells provided that these cells have the same sensitivity as the murine L1210 leukemic cells to this drug. The presence of significant amounts of uridine or cytidine in the body fluids should antagonize the cytotoxic action of 5-aza-C by decreasing the extent of phosphorylation of this nucleoside analog by uridine-cytidine kinase.

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