Cytotoxicity and Mode of Action of 5-Azacytidine on L1210 Leukemia


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SUMMARY

This study attempts to determine the primary actions of 5-azacytidine (5-azaCR) on L1210 leukemia. This agent was cytotoxic toward L1210 cells growing in culture with 50 and 90% inhibition dose values of 0.019 and circa 0.15 µg/ml, respectively. 5-AzaCR inhibits the incorporation of tritiated thymidine or deoxyadenosine into DNA to a greater extent than it inhibits the incorporation of tritiated uridine into RNA. Similar results were obtained with ascitic cells isolated from leukemic mice. Equimolar amounts of cytidine reduced the inhibition of DNA synthesis, as well as the inhibition of cell growth in culture caused by 5-azaCR. Uridine, but not deoxycytidine or deoxyuridine, was also effective, but to a lesser extent than was cytidine. With cell-free extracts isolated from L1210 cells in culture, no significant effect was found on enzyme systems directly or indirectly involved in DNA synthesis.

With 5-azaCR-4-14C as the precursor, this agent was found to be phosphorylated in all leukemic tissues studied. The majority of phosphorylated products existed as the triphosphate in ascitic and cultured L1210 cells. A portion (10 to 20%) of all these phosphorylated derivatives appeared to be further reduced to deoxyribonucleoside di- and/or triphosphate forms. 5-AzaCR was also incorporated into polynucleotides in all tissues studied and incorporated into both RNA (80 to 90% total incorporated radioactivity) and DNA fractions (10 to 20%) in L1210 cells in culture. In the presence of cytidine, phosphorylation of 5-azaCR, subsequent reduction, and incorporation into polynucleotides were greatly inhibited.

A probable mechanism of action of 5-azaCR on L1210 leukemia is proposed.

INTRODUCTION

5-AzaCR2 (NSC 102816) was chemically synthesized by Piskala and Sorm (23) in 1964. It was also isolated from the fermentation beer of Streptoverticillium ladakanus and identified in the Upjohn research laboratories (13). In addition to its antimicrobial activity, 5-azaCR showed pronounced cancerostatic effect in vivo against L1210 leukemia (13), AKR mouse leukemia (34), and Ehrlich ascites tumor (32). This compound has multiple effects on mammalian tissues. 5-AzaCR was found to be phosphorylated and incorporated into the RNA of several tissues studied (5, 6, 14, 17, 24, 26), with subsequent inhibition of RNA synthesis and disruption of many metabolic processes (5, 6, 15, 17). The inhibition by 5-azaCR of the decarboxylation of orotidylic acid and de novo pyrimidine synthesis in AKR mice (25), as well as the inhibition of DNA synthesis in primary rhesus monkey kidney cells in tissue cultures (33), has been reported. This report describes studies on the mode of action of 5-azaCR on L1210 cells, both in vivo and in culture.

MATERIALS AND METHODS

Tumor Lines. L1210 cells used for in vitro studies were grown at 37° in basal medium (100 parts of RPMI 1634 medium3 and 5 parts of fetal calf serum) developed by Moore et al. (22). A mixture of penicillin (0.1 µg/ml of medium) and streptomycin (0.5 µg/ml of medium) was added. Cells used in uptake studies were in the logarithmic phase of growth (circa 5 X 10^6 cells/ml).

Inbred female BDF [(C57BL x DBA/2] F1] mice (20 g ±3) used for in vivo studies were obtained from Jackson Memorial Laboratories, Bar Harbor, Maine. Mice received an i.p. injection of 1 X 10^6 ascitic L1210 cells and were routinely used in experiments when bearing approximately 1 X 10^8 cells (7 days after injection).

Chemicals and Medium. 5-AzaCR-4-14C (4 mCi/mmol) was obtained through the Chemical and Drug Procurement Section, Chemotherapy, National Cancer Institute, from Monsanto Research Corporation, Dayton Laboratories, Dayton, Ohio. TdR-3H (2 Ci/mmol), UR-3H (2 Ci/mmol), AR-3H (9.1 Ci/mmol), and AdR-3H (500 mCi/mmol) were purchased from New England Nuclear Corporation, Boston, Mass., and used without further purification. 5-AzaCdR was a gift from Dr. R. K. Robins, University of Utah, Salt Lake City.

"Some abbreviations used are: 5-azaCR, 5-azacytidine; TdR, thymidine; UR, uridine; AR, adenosine; AdR, deoxyadenosine; 5-azaCdR, 5-aza-2'-deoxycytidine; CR, cytidine; CDR, deoxycytidine; ID₉₀, 50% inhibition dose; ID₉₀, 90% inhibition dose; TCA, trichloroacetic acid; PCA, perchloric acid.

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City, Utah. 5-AzaCR was supplied by Aldrich Chemical Co., Inc., Milwaukee, Wis. CR, CdR, UR, uracil, orotic acid, AR, and guanosine were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Cytosine, deoxyuridine, orotidine, AdR, deoxyguanosine, and DNase were obtained from CalBiochem, Los Angeles, Calif. RNase was obtained from Sigma Chemical Company, St. Louis, Mo., and snake venom was obtained from General Biochemicals, Chagrin Falls, Ohio. RPMI 1634 medium and supplements were purchased from Grand Island Biological Co., Grand Island, N. Y.

L1210 Cell Growth Study. Twenty ml of cells (circa 1 X 10^8 cells/ml) were pipetted into sterilized culture tubes with screw caps and incubated at 37° overnight. The experiment was initiated by the addition of 1 ml of 5-azaCR (or medium) for a given period (from 0 to 240 min) prior to the addition of 1 ml of metabolite (or medium). Cell growth was determined twice a day for 3 days by means of a Model A Coulter counter (Coulter Electronics, Chicago, Ill.).

To determine ID_{50} and ID_{90} values, 5 ml of L1210 cells (5 X 10^6 cells/ml) were incubated with the drug at 37° for 3 days, and cell growth was determined (3).

Macromolecular Synthesis. The effect of 5-azaCR on L1210 cells was in part determined by evaluating its influence on the incorporation of labeled metabolites (Tdr-3H, UR-3H, Ar-3H, or Adr-3H) into respective macromolecules. L1210 cells were preincubated at 37° with 5-azaCR for up to 2 hr with continual gentle shaking. The reaction was started by the addition of labeled metabolite. Aliquots taken at different times were then pipetted into a centrifuge tube containing 1 ml of the corresponding unlabeled metabolite at high concentration (10 mg/ml) kept at 4°. Cell pellets were collected after centrifugation.

For the in vivo experiments, leukemic mice (bearing circa 1 X 10^8 cells/animal) were given injections i.p. with 0.2 ml of 5-azaCR of a given concentration. Two hr later, the reaction was started by injecting 0.5 ml of labeled metabolite (Tdr-3H or UR-3H, 10 μCi/12.5 μg). After 1 hr, 3 mice/group) were killed by cervical fracture, and the ascites were treated with heparin, collected, pooled, and then centrifuged at 4°. Supernatants were lyophilized and redissolved in a small volume of water just prior to paper chromatography.

Acid-insoluble fractions (DNA and RNA) were obtained by extracting the washed L1210 cell pellets twice with 1.5 ml of 0.5 N PCA at 70° for 20 min and mouse liver and spleen tissue pellets were washed with TCA and organic solvents. Acid-soluble fractions were isolated from washed L1210 cell pellets by 3 extractions with 1.5 ml of ice-cold 0.2 N PCA and from the erythrocyte-removed ascitic cells by 4 extractions with 2.5 ml of 0.2 N PCA (4°). Perchlorate was then removed as potassium perchlorate from all acid-soluble fractions by neutralization with KOH and centrifugation. Supernatants were lyophilized and redissolved in a small volume of water just prior to paper chromatography.

Enzyme Assays. A crude cell-free extract was isolated from L1210 cells in culture by suspension of the cells in a given volume of 0.05 M Tris-HCl buffer, pH 7.4, and sonic extraction with a Biosonik (Bronwill, Rochester, N. Y.) at 70% maximal output for 30 sec. The supernatant was collected after centrifugation at 105,000 X g for 60 min (4°) in a Model L Spinco ultracentrifuge. The final protein concentration of the cell-free extracts was approximately 3 mg/ml, determined by the procedure of Lowry et al. (20).

The extracts were used as the source of enzymes. Ribonucleotide reductase activity was measured according to the procedure of Turner et al. (35). A unit of enzyme is defined as the amount that catalyzed dCMP synthesis at a rate of 1 μmole/hr. For the determination of DNA polymerase activity, the method of Magee (15) was used. The assay systems for the measurement of pyrimidine nucleoside (CR) and deoxynucleoside (TdR, CdR) kinases were essentially those described by Chu and Fischer (4). However, reactions were terminated by heating for 2 min in a boiling water bath, and the phosphorylated derivatives were isolated according to the method of Bach (1). Fifty-μl aliquots were applied to 1-inch discs of diethylnitrosoethyl paper (H. Reeve Angel & Co., Clifton, N. J.), which were then placed in counting vials and eluted with 0.5 ml of 0.5 M PCA. After 1 hr, 12 ml of Diotol were added, and the radioactivity was determined.

Anabolic Transformation of 5-AzaCR. For the in vitro experiments, 38 ml of cell suspension (circa 5 X 10^5 cells/ml) were incubated at 37° with 1 ml of 5-azaCR-4-14C (4.8 μCi/1.2 μmoles) with or without the addition of 1 ml of CR or CdR (0.4 mg/ml). Immediately after incubation, cells were centrifuged at 800 X g for 10 min (4°) in 50-ml polyethylene tubes, then washed twice by resuspensions in 10 ml of fresh medium.

For the in vivo experiments, leukemic mice were given i.p. injections of 0.2 ml of 5-azaCR-4-14C [1 and 5 mg/kg (1.66 μCi/mouse)] with or without the simultaneous injection of CR (50 mg/kg). At the end of 1 to 4 hr, the animals (3 mice/group) were sacrificed, and the ascites were treated with heparin, collected, pooled, and then centrifuged at 4° and 800 X g for 10 min. Supernatants were saved for determination of radioactivity and chromatography. Erythrocytes were removed (12) by suspending the cell pack in 2 ml of 0.9% NaCl solution, adding 6 ml of distilled water (4°), mixing vigorously for 30 sec, adding 2 ml of 3.5% NaCl solution, and finally centrifuging at 160 X g for 15 min (4°). The cells were then washed twice with 10 ml of 0.9% NaCl solution.

Acid-soluble fractions were isolated from washed L1210 cell pellets by 3 extractions with 1.5 ml of ice-cold 0.2 N PCA and from the erythrocyte-removed ascitic cells by 4 extractions with 2.5 ml of 0.2 N PCA (4°). Perchlorate was then removed as potassium perchlorate from all acid-soluble fractions by neutralization with KOH and centrifugation. Supernatants were lyophilized and redissolved in a small volume of water just prior to paper chromatography.

After the removal of the acid-soluble fraction, the cell or tissue pellet was washed with TCA and organic solvents. Acid-insoluble fractions (DNA and RNA) were obtained by extracting the washed L1210 cell pellets twice with 1.5 ml of 0.5 N PCA at 70° for 20 min and mouse liver and spleen pellets at 100° for 45 min.
Separation of RNA from DNA. The procedure of Schmidt and Thannhauser (28) for separating RNA from DNA was modified with polynucleotides containing either TdR-3H or UR-3H as standards. The optimal condition for the hydrolysis of RNA of L1210 cells in culture is a 1-hr incubation with gentle shaking at 37° in 0.3 N KOH (unit weight of tissue/10 unit volume of KOH). At the end of incubation, 0.2 volume of 1.8 N HCl, 1 volume of DNA (1 mg/ml), and 0.8 volume of ice-cold 30% TCA were added sequentially to the system. The mixture was centrifuged in a Sorvall refrigerated centrifuge, Model R2C-B, at 3000 x g for 10 min at 4°, and the supernatant was used for radioactivity and RNA determinations. The precipitate containing the DNA fraction was then washed twice with 5 ml of cold 10% TCA and extracted twice with 1.5 ml of 0.5 N PCA at 70° for 20 min.

Hydrolysis of Acid-soluble and Acid-insoluble Fractions following 5-AzaCR-4-14C Incorporation. Lyophilized acid-soluble fractions were incubated with an equal volume of snake venom (50 µg/ml in 0.02 M Tris buffer, pH 9.0) at 37° for 4 hr.

Acid-insoluble fractions (washed cell pellets) were incubated with a mixture of DNase (1 mg/ml) and RNase (1 mg/ml) at 37° for 1 hr. An equal volume of snake venom (50 µg/ml) was then added, and incubation was continued for 4 hr. Protein was precipitated with 10% PCA, and the perchlorate was then removed by neutralization with KOH and centrifugation. Supernatants were lyophilized and redissolved in a small volume of water just prior to thin-layer chromatography.

Chromatography. Acid-soluble fractions were separated by paper chromatography (Whatman No. 2 paper) using two solvent systems. The first system (isobutyric acid:H2O:concentrated NH4OH in a 50:30:1 ratio, pH 3.6) will separate a nucleoside from its corresponding mono-, di-, and triphosphate derivatives. The second system [5 M ammonium acetate (pH 9.5):saturated sodium borate:95% ethanol:0.5 M perchlorate] will separate a deoxyribonucleoside and its phosphorylated derivatives from corresponding compounds containing a ribosyl moiety. 5-AzaCR and both CR- and CdR-5'-mono-, di-, and triphosphate were used as standards in both systems. In order to determine the radioactivity distribution of the chromatograms, dried sheets were examined under ultraviolet light, and the areas occupied by standards were noted. Strips (1.25 x 32 inches) were cut into 1-inch sections, the sections were quartered, and the pieces were placed in a scintillation counting vial. After a 2-hr elution period, 12 ml of Diotol were added, and the vials were counted. Strips (1.25 x 32 inches) were cut into 1-inch sections, the sections were quartered, and the pieces were placed in a scintillation counting vial. After a 2-hr elution period, 12 ml of Diotol were added, and the vials were counted.

Hydrolyzates of acid-soluble and acid-insoluble fractions following 5-azaCR-4-14C incorporation were separated on cellulose plates (Brinkman Instruments, Inc., Westbury, N. Y.) and developed with the borate solvent (27) for approximately 6 hr. Sections (1 x 3 sq cm) were scraped from the plates, eluted with 0.5 ml of 0.5 N PCA, and counted in Diotol.

RESULTS

Cytotoxicity. Cytotoxicity against L1210 cells in culture was determined by the method of Buskirk (3). When 5-azaCR and cells were in contact for 3 days (37°), the ID50 and ID90 values were 0.019 and circa 0.15 µg/ml, respectively.

Effect of 5-AzaCR on Macromolecule Synthesis in L1210 Cells in Culture. Data presented in this report were derived from linear rates of incorporation obtained for incubation periods up to 60 min at 37°.

As illustrated in Chart 1, DNA synthesis (TdR-3H incorporation) is inhibited by 5-azaCR to a greater extent than RNA synthesis (UR-3H incorporation). After a 90-min preincubation, TdR-3H incorporation is inhibited about 74%, as compared to only 32% inhibition of UR-3H incorporation. To maximize the effect of 5-azaCR, a 2-hr preincubation period and a subsequent incorporation period of up to 1 hr were routinely used in succeeding experiments.

The results of a dose-response study of the effect of 5-azaCR on polynucleotide synthesis are summarized in Chart 1. The ID50 for TdR-3H incorporation is about 3.5 µg/ml; for UR-3H incorporation, it is about 10 µg/ml. 5-AzaCR was also found to inhibit the incorporation of purine metabolites into macromolecules. In the presence of 5 µg/ml of 5-azaCR, little or no effect was observed on AR-3H incorporation, but the rate of AdR-3H incorporation into polynucleotides is inhibited about 50% for the same period.

Effect of 5-AzaCR on Polynucleotide Synthesis in Leukemic BDF1 Mice. In order to compare its action in vivo and in vitro, the effect of 5-azaCR on the incorporation of TdR-3H and UR-3H into respective macromolecules of ascitic cells of leukemic BDF1 mice was studied. TdR-3H incorporation was significantly inhibited when the animals...
Table 1

Inhibition of macromolecular synthesis in leukemic L1210 cells in culture by 5-azaCR

L1210 cells (circa 5 × 10⁶ cells/ml) were preincubated with 5-azaCR at 37°C for 2 hr; the reaction was started by adding labeled metabolite, e.g., TdR-3H, UR-3H, AdR-3H, or AR-3H (1 μg/ml of cells) and incubating for up to 60 min (within linear rate of incorporation and the S.D. of 5%). The acid-insoluble fractions were obtained by extracting the washed cell pellet twice with 1 ml of 0.5 N PCA for 20 min at 70°C and were used for counting radioactivities and determining the nucleic acid contents.

<table>
<thead>
<tr>
<th>Inhibition (%)</th>
<th>5-azaCR concentration (μg/ml)</th>
<th>DNA synthesis</th>
<th>RNA synthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TdR-3H</td>
<td>AdR-3H</td>
<td>UR-3H</td>
</tr>
<tr>
<td>1.0</td>
<td>14</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>2.5</td>
<td>41</td>
<td>37</td>
<td>15</td>
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<td>5.0</td>
<td>65</td>
<td>50</td>
<td>34</td>
</tr>
<tr>
<td>10.0</td>
<td>78</td>
<td>63</td>
<td>58</td>
</tr>
<tr>
<td>25.0</td>
<td>78</td>
<td>78</td>
<td>78</td>
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</tbody>
</table>

*Calculated from cpm or specific activity (cpm/μg of DNA or RNA) of controls.

were exposed to 5-azaCR for 2 hr or longer; therefore, a 2-hr preincubation with this agent and a 1-hr incorporation period were chosen as routine *in vivo* assay conditions.

The results, shown in Table 2, indicate that in ascitic cells the incorporation of TdR-3H into DNA is inhibited to a greater extent than that of UR-3H into RNA. Thus, at the 25-mg/kg level, DNA synthesis is inhibited approximately 50%, as compared to only a 15% inhibition of RNA synthesis. This corresponds closely to the *in vitro* situation as reported in the previous section.

**Effect of Various Pyrimidines and Pyrimidine and Purine Nucleosides on 5-AzaCR Inhibition of DNA Synthesis and L1210 Cell Growth in Culture.** The inhibition of TdR-3H incorporation by 5-azaCR was completely prevented by the simultaneous addition of approximately equimolar (20 μM or 5 μg/ml) CR (Table 3) and partially prevented by UR. Neither CdR, deoxyuridine, orotidine, cytosine, uracil, orotic acid, AR, AdR, guanosine, nor deoxyguanosine had any significant effect. In addition, the inhibition of AdR-3H incorporation by 5-azaCR was also prevented by the presence of approximately equimolar CR or UR.

Table 3

Effect of various metabolites on the inhibition of DNA synthesis by 5-azaCR in L1210 cells in culture

L1210 cells (circa 5 × 10⁶ cells/ml) were preincubated with the simultaneous additions of 5-azaCR (or medium) and a given metabolite (or medium) at 37°C for 2 hr. The reaction was started by adding TdR-3H (1 μg/ml of cells) and incubating for up to 60 min at 37°C. The acid-insoluble fractions were obtained by extracting the washed cell pellet twice with 1 ml of 0.5 N PCA for 20 min at 70°C.

<table>
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<tr>
<th>Experiment</th>
<th>Specific activity (cpm/μg of DNA)</th>
<th>Control TdR-3H</th>
<th>CR</th>
<th>CdR</th>
<th>UR</th>
<th>Deoxyuridine</th>
<th>AdR</th>
<th>Guanosine</th>
<th>Deoxyguanosine</th>
<th>AR</th>
<th>AdR</th>
<th>Uracil</th>
<th>Orotic acid</th>
<th>Orotidine</th>
</tr>
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<tr>
<td>1</td>
<td>204</td>
<td>113</td>
<td>45</td>
<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>79</td>
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<tr>
<td>2</td>
<td>914</td>
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<td>2</td>
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<td>68</td>
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<td>126</td>
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<td>0</td>
<td>0</td>
<td>62</td>
</tr>
</tbody>
</table>

*aCalculated from the specific activity (cpm/μg of DNA or RNA) of controls.

Effect of 5-AzaCR on the Enzyme Systems of Crude Cell-Free Extracts of L1210 Cells in Culture. The effect of 5-azaCR on enzymes directly or indirectly involved in DNA synthesis, e.g., TdR kinase, CdR kinase, ribonucleotide reductase, and DNA polymerase, was studied. 5-AzaCR at 5 μg/ml or higher concentrations showed no significant effect on any of the enzyme systems in cell-free extracts of L1210 cells.
Chart 2. Effect of various metabolites on the inhibition of L1210 cell growth in culture by 5-azaCR. Twenty ml of cells (circa 1 x 10⁴ cells/ml) were pipetted into sterilized culture tubes fitted with screw caps and incubated at 37° overnight. The experiments were initiated by the simultaneous addition of equimolar concentrations (final concentration, 20 µM or 5 µg/ml) of 5-azaCR and various metabolites, e.g., CR, UR, etc., and incubated at 37° for up to 70 hr. Cell growth was determined twice daily by means of a Model A Coulter counter; 3 samples were averaged.

Anabolic Transformation of 5-AzaCR. 5-AzaCR was phosphorylated in LI210 cells in vivo and in culture. The majority of phosphorylated 5-azaCR derivatives in the acid-soluble fractions moved with the same Rf as the triphosphate form (Chart 4). Chromatography of the acid-soluble fraction in the borate system gave 3 major peaks, as illustrated in Chart 5. In addition to the 5-azaCR peak, 1 large peak is seen near the origin which corresponds in mobility to a CMP, CDP, and CTP region, again indicating this antimetabolite is phosphorylated in L1210 cells in vivo and in culture. The 2nd peak seen reproducibly moves with the dCDP and/or dCTP region and represents 10 to 20% of the radioactivity of the larger peak. When acid-soluble fractions were incubated with snake venom phosphodiesterase, 5-azaCdR was found to be present in the hydrolysate (Chart 6), indicating that this 2nd small peak represents deoxyribonucleotide derivatives of 5-azaCR.

In the presence of an approximately equimolar amount of CR, both the phosphorylation of 5-azaCR and subsequent reduction are inhibited over 90% in L1210 cells in culture (Charts 4 and 5) and are also significantly reduced in the in vivo situation (Chart 5). The amount of 5-azaCR present in the cells, however, is unaltered (Charts 4 and 5).

When 5-azaCR was incubated with L1210 cells for 1 hr at 37°, the radioactivity contained in the medium was present as a single peak corresponding with the 5-azaCR standard. Apparently, 5-azaCR did not decompose significantly during the incubation period. In ascitic fluid isolated from leukemia BDF₁ mice, more than 1 radioactive peak appeared to be present at the end of 4 hr of incubation. However, the major

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4UR kinase represents CR and/or UR kinase which has a nearly equal affinity for CR and UR in L1210 leukemia.
Table 4

Effect of 5-azaCR on the enzyme systems of crude cell-free extracts isolated from L1210 cells in culture

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Control (no drug)</th>
<th>5-azaCR (μg/ml)</th>
<th>Inhibition (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>cpm/min</td>
<td>cpm/min</td>
<td></td>
</tr>
<tr>
<td>DNA polymerase</td>
<td>13.97 ± 1.22</td>
<td>12.19 ± 1.06</td>
<td>~13</td>
</tr>
<tr>
<td></td>
<td>13.97 ± 1.22</td>
<td>13.07 ± 0.43</td>
<td>~6</td>
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<tr>
<td></td>
<td>7.49 ± 0.41</td>
<td>6.91 ± 0.4</td>
<td>~8</td>
</tr>
<tr>
<td>TdR kinase</td>
<td>10.23 ± 0.91</td>
<td>11.11 ± 0.72</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>9.37 ± 1.23</td>
<td>9.17 ± 0.93</td>
<td>~2</td>
</tr>
<tr>
<td>CDR kinase</td>
<td>10.68 ± 0.63</td>
<td>11.04 ± 0.81</td>
<td>0</td>
</tr>
<tr>
<td>UR kinase</td>
<td>6.49 ± 0.57</td>
<td>6.37 ± 0.33</td>
<td>~2</td>
</tr>
<tr>
<td></td>
<td>6.49 ± 0.57</td>
<td>6.88 ± 0.36</td>
<td>0</td>
</tr>
<tr>
<td>Ribonucleotide reducse</td>
<td>1.44 units/mg protein</td>
<td>1.65 units/mg protein</td>
<td>0</td>
</tr>
</tbody>
</table>

*aThe enzyme rates (cpm/min) were derived from the slope of initial incorporation rates corrected via linear regression program on a Model 9100 Hewlett-Packard calculator. The enzyme rates of controls varied from experiment to experiment because of the different preparations of cell-free extract used.

*bThe assay and work-up conditions for DNA polymerase were essentially those described by Magee (21). The reaction mixture contained (in μmoles): potassium phosphate (pH 7.4), 15; EDTA, 2; ATP, 0.05; Mg++, 2.5; triphosphates of CDR, deoxyguanosine, and AdR, 0.01 each; TdR triphosphate-3H, 0.01 (100 μCi/m mole); DNAse-treated E, coli DNA, 0.2 mg; and cell sonicate, 0.2 mg protein in a total volume of 0.5 ml. After incubation at 37° for up to 60 min with continual gentle shaking, the reactions were stopped by the addition of carrier DNA (200 μg) and PCA (to 0.5 N) containing 2% Celite. The pellet was washed with PCA, dissolved in 1 N NaOH, reprecipitated and washed with PCA, extracted with 0.15 ml 0.5 N PCA (70°) for 20 min, and counted in 12 ml of Diotol.

cThe assay conditions for nucleoside and deoxynucleoside kinases are essentially those described by Chu and Fischer (4) for CDR kinase. The complete reaction mixture in 0.2-ml final volume contained ATP, 2.25 X 10⁻³ M; MgCl₂ 3.5 X 10⁻³ M; the appropriate substrate (TdR-3H, etc.), 0.0015 μmoles; protein (crude cell-free extract) 0.2 mg; Tris buffer, 10.0 μmoles (pH 7.95). The reaction system was incubated at 37° for up to 60 min with continual gentle shaking and terminated by heating for 2 min in a boiling water bath. Fifty-μl aliquots were applied to 1-inch discs of diethylaminoethyl paper (1). The discs were thoroughly washed with distilled water on a fritted glass funnel under suction, followed by rinses with ethanol. The phosphorylated derivatives of either the nucleosides or deoxynucleosides were extracted by the addition of 0.5 ml of 0.5 M PCA to the discs in counting vials. This was immediately followed by the addition of 12 ml of Diotol.

dThe assay mixture (0.25 ml) for ribonucleotide reductase, according to Turner et al. (35), contained 20 μM histidine buffer (pH 6.9), 5 mM dithiothreitol, 8 mM NaF, 10 mM MgCl₂, 5.5 mM ATP, 0.34 to 0.51 mM labeled CMP (approximately 10⁷ cpm/mole), and freshly prepared soluble extract (0.5 to 4 mg of protein). After 30 min at 37°, the mixtures were chilled in ice and diluted to 1.0 ml with carrier dCMP (2.3 μmoles) and PCA (final concentration, 0.5 M). After removal of insoluble material by centrifugation, the acid solutions were kept for 15 min at 100° to convert the cytosine nucleotides to monophosphates. The PCA was removed by precipitation with 1 N KOH, the neutralized solutions were reacidified with acetic acid to a final concentration of 0.2 M, and dCMP was isolated by ion exchange chromatography with Dowex 50 (acid form). An automated bank of columns packed with Dowex 50–H⁺ (13 x 1 cm) and eluted with 0.2 M acetic acid was used for this purpose. They were fitted with timer-operated solenoid controls to divert all of the eluent to waste, except for the dCMP fraction (from 195 to 270 ml). The dCMP fractions were concentrated, and the radioactivity was determined in a scintillation counter. For this purpose, aqueous solutions (1 ml) were added to phosphor solutions in dioxane (15 ml). The observed counts were corrected for the recovery of dCMP carrier, as estimated by absorbance measurements at 280 μm.

Portion still existed as 5-azaCR. Since the pyrimidine nucleoside deaminase level has been found to be very low in leukemic L1210 cells (7, 10), the deamination of 5-azaCR will probably not play an important role in the anabolic transformation of this antimetabolite.

5-AzaCR was also incorporated into the polynucleotides of liver, spleen, and ascitic cells of leukemic mice. In the presence of CR (50 mg/kg), incorporation in all tissues was greatly inhibited. As can be seen in Table 5, the inhibition by CR was most pronounced in ascitic cells. 5-AzaCR was incorporated into both RNA (circa 80 to 90% total incorporated radioactivity) and DNA (circa 10 to 20%) fractions isolated from L1210 cells in tissue culture (Table 6), and the rate of incorporation was nearly linear for at least 1 hr (37°). Identification of 5-azaCdR in the hydrolysate of the washed cell pellets (Chart 6) was further evidence of 5-azaCR incorporation into DNA. In the presence of approximately equimolar CR, 5-azaCR incorporation into both DNA and RNA fractions was nearly completely inhibited.
DISCUSSION

5-AzaCR was found to be active against L1210 cells in vivo (13) and in culture. In attempting to understand the nature of its activity, the effect of 5-azaCR on macromolecular synthesis was studied. For the same period of incubation, the inhibition of DNA synthesis appears to be an earlier and stronger event than that of protein synthesis, but the inhibition of protein synthesis quickly approaches the degree of inhibition of DNA synthesis (L. H. Li, unpublished data). Inhibition of polynucleotide synthesis by 5-azaCR may subsequently be responsible for inhibition of protein synthesis, as in studies on isolated nuclei of calf-thymus cells reported by Raskas et al. (24), but the significance of this inhibition of protein synthesis remains to be determined.

As seen in Tables 1 and 2, 5-azaCR inhibits DNA synthesis more than RNA synthesis in L1210 cells in vivo and in culture. This phenomenon has also been observed by Sorm et al. (33) in rhesus monkey cells in tissue culture, but has not yet been extensively studied in AKR mouse leukemia and Ehrlich ascites tumors. The inhibition of RNA synthesis by 5-azaCR may play a major role in the decrease of acceptor activity of tRNA (15), the breakdown of polyribosomes (5, 17), the incorporation into mRNA causing the subsequent inhibition of protein synthesis (24), and enzyme inductions (6). However, the marked inhibition of DNA synthesis in L1210 leukemia cannot be ignored as a main site of biochemical action of this compound.

The relatively high concentration required to reduce the macromolecular synthesis to one-half as compared to the ID₅₀ for the cell growth may be due to the long period of incubation (3 days at 37°) before the inhibition of cell growth was determined. However, by comparing the inhibition of DNA synthesis and that of mitosis of L1210 cells in culture for the same period of incubation, the results are rather compatible (19).

Results summarized in Table 1 show that 5-azaCR inhibits the incorporation of both AdR-3H and TdR-3H into DNA. Since AdR shows no effect on the inhibition of TdR-3H incorporation by 5-azaCR (Table 3) and because of its pyrimidine structure, 5-azaCR probably inhibits AdR-3H incorporation by inhibiting the final stages of DNA synthesis rather than by inhibiting the enzymes involved in the phosphorylation of AdR-3H. Table 4 illustrates that even at the higher concentrations (5 to 50 μg/ml) 5-azaCR showed no apparent effect on TdR kinase, CdR kinase, and ribonucleotide reductase, again indicating that the site of 5-azaCR inhibition may be the final stage of DNA synthesis.

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Although the possible influence of the metabolic products of 5-azaCR, e.g., the S'-triphosphate of 5-azaCdR, on DNA polymerase cannot be completely ruled out, the lack of an effect of relatively higher concentrations (5 to 50 μg/ml) of 5-azaCR on DNA polymerase suggests that the inhibition of DNA synthesis by 5-azaCR is likely to be the result of the incorporation of this agent into DNA and prevention of further synthesis.

Table 5

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Acetic cells</th>
<th></th>
<th></th>
<th>Liver</th>
<th></th>
<th></th>
<th>Spleen</th>
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<th></th>
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<tbody>
<tr>
<td>Specific activity</td>
<td>inhibition (%)</td>
<td>Specific activity</td>
<td>inhibition (%)</td>
<td>Specific activity</td>
<td>inhibition (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60 min -CR</td>
<td>5,610</td>
<td>0</td>
<td>1,670</td>
<td>46</td>
<td>1,480</td>
<td>60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+CR</td>
<td>1,080</td>
<td>81</td>
<td>908</td>
<td>0</td>
<td>599</td>
<td>0</td>
<td></td>
<td></td>
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<tr>
<td>130 min -CR</td>
<td>28,600</td>
<td>0</td>
<td>5,780</td>
<td>0</td>
<td>4,220</td>
<td>0</td>
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<td></td>
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<td>95</td>
<td>1,220</td>
<td>79</td>
<td>1,200</td>
<td>72</td>
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</tr>
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</table>

*Specific activity, cpm/mg RNA.

Table 6

<table>
<thead>
<tr>
<th>Treatment</th>
<th>RNA fraction</th>
<th>DNA fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific activity</td>
<td>Inhibition (%)</td>
<td>Specific activity</td>
</tr>
<tr>
<td>0 min control</td>
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<td>0.21</td>
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<td>15 min control</td>
<td>7.63</td>
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<td>8.13</td>
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<tr>
<td>30 min + CR (10 μg/ml)</td>
<td>0.06</td>
<td>99</td>
</tr>
<tr>
<td>60 min + CR (10 μg/ml)</td>
<td>1.08</td>
<td>96</td>
</tr>
</tbody>
</table>

*Concentration of 5-azaCR-4-14C, 7.25 μg/ml cells.

*Separation according to a modification of the procedure of Schmidt and Thannhauser; condition for hydrolysis of RNA is 0.3 N KOH at 37°C for 1 hr.

*Specific activity, cpm/μg DNA or RNA.

If, as proposed in the preceding section, the inhibition of DNA synthesis by 5-azaCR is the result of its incorporation into newly formed polynucleotides, we would expect that the compound must be phosphorylated to become biologically active. Chart 4 indicates that 5-azaCR is indeed phosphorylated in ascitic cells from leukemic mice, as well as in cultured L1210 cells. Charts 5 and 6 strongly suggest that the phosphorylated derivatives of 5-azaCR are further reduced to corresponding deoxyribonucleotides, presumably through the ribonucleotide reductase system (16), since the reduced products exist primarily in di- and/or triphosphate forms. Only traces of 5-azaCdR monophosphate were detected in the acid-soluble fractions of all the tissues studied. Therefore, 5-azaCR probably is not metabolized via the deoxycytidylate deaminase pathway in L1210 leukemic cells, though the level of deoxycytidylate deaminase in ascitic L1210 cells has been reported by Schrecker and Urshel (29) to be quite high.
To determine the enzyme systems responsible for the phosphorylation of 5-azaCR, the effect of various structurally related metabolites on the inhibition of DNA synthesis and cell growth in culture by 5-azaCR was studied. As would be expected, since they are generally regarded (30) as poor precursors for nucleic acid synthesis in mammalian systems, cytosine, uracil, and orotic acid showed little or no suppressive effect (Table 3). Moreover, orotidine, CdR, deoxyuridine, and purine nucleosides also showed no effect (Table 3; Chart 2), indicating that the phosphorylation of 5-azaCR is probably not through CdR kinase systems. On an equimolar basis, only CR and, to a lesser extent, UR prevented 5-azaCR inhibition of both DNA synthesis (Table 3) and cell growth (Chart 2) of L1210 cells in culture. These findings agree with those of Evans and Hanka (11) for L1210 cells in vivo and suggest that 5-azaCR is phosphorylated in L1210 leukemia via the UR kinase system as reported for AKR mouse leukemia and Ehrlich ascites tumor by Jurovšik et al. (14). This mechanism was further substantiated when, in the presence of CR, both phosphorylation of 5-azaCR and subsequent reduction, as well as its incorporation into both DNA and RNA, were markedly reduced. CdR had no significant effect.

Uridine kinase in Ehrlich ascites has been observed by Sköld (31) to have nearly equal affinity for CR and UR. If an analogous situation is present in leukemic L1210 cells, CR and UR compete with 5-azaCR for this kinase, which subsequently causes a decrease in the observed inhibitory activities of this agent. We failed, however, to show any effect of 5-azaCR on the phosphorylation of CR in cell-free extracts isolated from L1210 cells (Table 4). This could be due to the stronger affinity of UR kinase for the substrate (CR and/or UR) than for 5-azaCR.

Doskocil and Šorm (9) reported that the production of viable phage particles by Escherichia coli infected with bacteriophage T4 was inhibited by 5-azaCR. DNA synthesis in phage-infected E. coli was strongly inhibited by 5-azaCR, while RNA synthesis remained unaffected. As in our studies, inhibition of DNA synthesis was not prevented by CdR, but rather by CR. It is possible that 5-azaCR acts in a manner in L1210 cells similar to that in phage-infected E. coli.

As seen in Chart 3, the addition of a high concentration of CR (CR:5-azaCR in a ratio of 40:1) 20 min after incubation with 5-azaCR can no longer prevent the inhibitory effect of 5-azaCR. These observations are very interesting in the light of the results of the incorporation of 5-azaCR-4,14°C into intact L1210 cells (Table 6). A small but significant quantity of 5-azaCR-4,14°C is incorporated into both acid-soluble (mostly in triphosphate form) and acid-insoluble fractions in the first 15-min incubation (37°). These results collectively suggest that the inhibition of cell growth can no longer be completely prevented after 5-azaCR is phosphorylated and/or incorporated into macromolecules of L1210 cells.

The results of our experiments can be synthesized into a simple model (Chart 7). Antimetabolic activity in vivo and in vitro initiates with the conversion of 5-azaCR to corresponding 5'-polyphosphates through UR kinases and ribonucleotide reductase. Subsequent incorporation into RNA and DNA and resulting inhibition of nucleic acid synthesis is interpreted as the main cause of the marked antileukemic effect of this compound, although other effects of 5-azaCR on L1210 leukemia cannot be completely ruled out, e.g., the interference with the formation of pyrimidines de novo (25).

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