Incorporation of a Potent Antileukemic Agent, 5-Aza-2'-deoxycytidine, into DNA of Cells from Leukemic Mice

J. Veselý and A. Čihák

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6, Czechoslovakia

SUMMARY

5-Aza-2'-deoxycytidine administered at a daily dose of 1.5 mg/kg increased the life-span of P388 leukemia-bearing BALB/c × DBA/2 F1, mice by 5 times and that of second generation lymphoma-bearing AKR mice by 2.5 times. Higher doses (total dose, 20 mg/kg) led to favorable results when administered in two portions on Days 4 and 5 after the s.c. inoculation of leukemic cells. The same total dose given on 5 consecutive days was toxic. The lethal dose that killed 50% of the animals was 190 mg/kg. The drug was also effective in L1210 leukemia. 5-Aza-2'-deoxycytidine inhibited the phosphorylation of 2'-deoxyctidine in the acido-soluble pool of cells from leukemic AKR mice as well as its incorporation into DNA. In vitro the inhibition of the uptake of 2'-deoxycytidine into cells from leukemic mice by 5-aza-2'-deoxycytidine had a competitive character (K, 8 × 10⁻⁹ M). Although 5-aza-2'-deoxy[4-¹⁴C]cytidine of low-specific activity was not detected in DNA isolated from the lives of leukemic mice, the same tritium-labeled drug of high-specific radioactivity was selectively localized in the nuclei of leukemic cells as revealed by autoradiography. The incorporation of [³H]-5-aza-2'-deoxycytidine into DNA of cells from leukemic mice was confirmed by the chromatographic separation of DNA on a column of kieselguhr coated with methylated albumin.

INTRODUCTION

aza-dCyd¹ was prepared synthetically in this Institute by Plíml and Sorm (12). It is a potent antibacterial agent (3) and strongly inhibits the growth of hamster fibrosarcoma cells in vitro (9). Antileukemic properties of aza-dCyd have been reported in 2nd generation AKR mouse leukemia (14, 15). In plant chromosomes the drug causes local uncoupling of specific chromosome segments and chromatid aberrations (6).

In mouse tissues the main sites of attack of aza-dCyd are lymphatic organs and bone marrow, in which depression of the granular series is most marked on the 3rd day after drug administration (14). The mechanism of the inhibitory effect of aza-dCyd is not clear at present. By use of cytidine deaminase-deficient strains of Escherichia coli, it has been concluded that aza-dCyd enters the cell via deamination followed by phosphorolytic cleavage of the glycosidic bond (5). With [³H]-aza-dCyd of high-specific radioactivity, the preferential uptake of the drug in the lymphatic system of mice is observed (4). The aim of this report is to show optimal conditions of aza-dCyd administration during the treatment of mouse leukemia and to extend our knowledge about the inhibitory mechanism of this agent in cells of leukemic mice.

MATERIALS AND METHODS

Materials. 5-Azacytidine, aza-dCyd, and 5-aza-2'-deoxy[4-¹⁴C]cytidine (10 µCi/µmole) were prepared in the Department of Organic Synthesis of this Institute. [³H]-aza-dCyd (19.5 Ci/mmole) was prepared by Dr. B. Cenny, Isotope Laboratory, Prague-Krč, Czechoslovakia. [2-¹⁴C]-Thymidine (52 µCi/µmole) and 2'-deoxy[2-¹⁴C]cytidine (25 µCi/µmole) were obtained from the Institute for Research, Production and Uses of Radioisotopes, Prague, Czechoslovakia. The purity of radioactive compounds was verified chromatographically in a solvent system composed of isobutyric acid:ammonium hydroxide:water (66:1.5:33, v/v/v). DNase (bovine pancreas) was supplied by Calbiochem, Lucerne, Switzerland.

Animals. Female AKR and BALB/c × DBA/2 F1, hereafter called CD2F1, mice, kept under standard conditions, were used throughout the experiments. The ascitic tumors were implanted i.p. In the case of AKR mouse leukemia, infiltrated livers were homogenized in 0.15 M NaCl, and the resulting cell suspension was inoculated s.c. The inhibitors were dissolved in water and administered i.p. in a total volume of 0.2 ml.

Incorporation of 2'-Deoxycytidine and Cytidine into Liver DNA and RNA in Leukemic AKR Mice in Vivo. Groups of female AKR mice (25 g) during the terminal stage of leukemia received 2'-deoxy[2-¹⁴C]cytidine (1 µCi/µmole/animal) or [2-¹⁴C]cytidine (0.5 µCi/µmole/animal) i.p. 90 min before being killed. The excised livers were homogenized under cool conditions in 4 volumes of 0.15 M KCl. Aliquots of homogenates were mixed with an equal volume of cold 0.4 M HClO₄, the precipitates were centrifuged, and the supernatant fractions were separated chromatographically on Whatman No. 3 paper in isobutyric acid:ammonium hydroxide:water. Spots of 2'(3')-CMP were eluted and further purified in isopropyl alcohol:hydrochloric acid:water (170:41:39, v/v/v). After alkaline hydrolysis the sediments were evaporated until dry,
and hydrolysis of DNA (1 hr; 70% HClO4; 100°) was carried out. After neutralization the supernatant fractions were separated chromatographically on Whatman No. 3 paper in a solvent system composed of 1-butanol:acetic acid:water (10:1:3, v/v/v). Separated spots of cytosine were eluted and rechromatographed in a solvent system composed of isopropyl alcohol:ammonium hydroxide:water. Spectroscopically pure compounds were examined after elution with a Unicam SP 700 spectrophotometer; the radioactivity was assayed in a Packard liquid scintillation spectrometer. The incorporation of cytidine into RNA is expressed as the specific radioactivity of 2'(3')-CMP isolated from RNA (dpm/molecule), and the incorporation of 2'-deoxycytidine into DNA is expressed as the specific radioactivity of cytosine isolated from DNA (dpm/molecule).

Analysis of Acid-soluble Pool of Leukemic Cells from AKR Mice. A cell suspension from livers of leukemic AKR mice was prepared by gently homogenizing the tissue in Eagle's minimum essential medium enriched with 10% trypsin, 8% calf serum, and 2% embryonal bovine serum with a loose-fitting, hand-operated plastic pestle in a glass homogenizer under cool conditions. Cells were counted, and their number was adjusted to 10^7/ml. For the assay of metabolic conversion of 2'-deoxycytidine, the cell suspensions in a total volume of 5 ml were incubated 10 min at 37° in a Dubnoff incubator. 2'-Deoxy[2-14C]cytidine (0.1 mmol) was added simultaneously with aza-dCyd (0.5 mmol). After repeated washings of the cells, the pellets were extracted twice with ice-cold 0.2 M HClO4. The extracts were combined, neutralized, and separated chromatographically (Whatman No. 1 paper) in isobutyric acid:ammonium hydroxide:water. Radioactive 2'-deoxycytidine and corresponding 5'-phosphates were located according to the position of standards, and their radioactivity was measured with a Packard liquid spectrometer. The amount of newly formed uridine and of its derivatives was negligible. The results were expressed in dpm/Av. incorporation.

Incorporation of 2'-Deoxy[4-14C]cytidine into Cells from Leukemic Mice In Vitro. Cell suspensions were prepared as described above. Incubation at 37° in a Dubnoff shaking incubator lasted for 10 min. aza-dCyd was added simultaneously with 0.01 mm 2'-deoxy[2-14C]cytidine. After centrifugation and repeated extraction with cold 5% trichloroacetic acid, the sediments were treated for 20 min at 90° with 5% trichloroacetic acid. The radioactivity of extracts was expressed per 1 mg of total nucleic acids.

aza-dCyd and Thymidine Incorporation into DNA in Livers of Leukemic Mice In Vivo. Leukemic AKR mice were given [4-14C]aza-dCyd or thymidine (5 μCi/0.5 μmole/animal) or [3H]aza-dCyd (100 μCi/0.1 μmole/animal) i.p. 2 hr prior to being killed. The livers were removed and immediately homogenized at 4° in 5 volumes of 0.25 M sucrose with 1 M MgCl2. The homogenates were spun down and washed 3 times with cold 0.25 M sucrose. The sediments were suspended in 0.05 M Tris-HCl buffer (pH 8.0) containing 0.01 M EDTA, 0.15 M NaCl, and 0.015 M sodium citrate. Guanidine hydrochloride (pH 6.0) was added to a final concentration of 4 M (10), and the samples were mixed for 30 min at 4°. DNA was precipitated with ethanol and dissolved in 0.015 M NaCl with 0.0015 M sodium citrate (pH 7.0); afterward the concentration of these salts was adjusted to 0.15 M and 0.015 M, respectively. The chromatography of DNA was done on a methyalted albumin-kieselguhr column (3 x 3 cm) prepared as described by Mandell and Hershey (8). DNA (1 to 2 mg) was eluted with a linear gradient of 0.4 to 1.0 M NaCl in 50 mM phosphate buffer (pH 6.7), and fractions of 2.0 ml were collected.

Autoradiography of Cells from Leukemic Mice with [3H]Thymidine and [3H]aza-dCyd. [methyl-3H]Thymidine (200 μCi/0.025 μmole/animal) or [3H]aza-dCyd (200 μCi/0.025 μmole/animal) i.p. were administered to groups of leukemic AKR mice 2 hr before they were killed. Smears from livers and inguinal lymph nodes were done on gelatinized glass slides, and the preparations were immediately fixed (3 min) in cold methanol. Some of the slides were treated with 5% trichloroacetic acid (3 min; 4°), with DNase [50 μg per ml of 50 mM Tris-HCl buffer (pH 7.4) containing 0.2 M MgCl2] for 24 hr at 37° or with the buffer only. Afterward, they were thoroughly washed in water and, after being coated with a stripping film (Kodak AR. 10), they were exposed at —20° for 2 weeks. After development the slides were stained with Giemsa, and the grains were counted for each slide over 500 cells. The background was negligible.

RESULTS

The effect of different doses of aza-dCyd on mouse leukemia P388 and L1210 is shown in Table 1. In P388 leukemia

<table>
<thead>
<tr>
<th>Daily dose (mg/kg)</th>
<th>P388 Av. surv. (days)</th>
<th>% surv.</th>
<th>Av. weight change (g)</th>
<th>L1210 Av. surv. (days)</th>
<th>% surv.</th>
<th>Av. weight change (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>14.0</td>
<td>140</td>
<td>—0.2</td>
<td>13.0</td>
<td>186</td>
<td>—0.8</td>
</tr>
<tr>
<td>0.4</td>
<td>18.0</td>
<td>160</td>
<td>+0.7</td>
<td>12.5</td>
<td>179</td>
<td>—0.4</td>
</tr>
<tr>
<td>0.8</td>
<td>25.5</td>
<td>255</td>
<td>—1.0</td>
<td>9.5</td>
<td>136</td>
<td>—1.2</td>
</tr>
<tr>
<td>1.6</td>
<td>25.0</td>
<td>250</td>
<td>—0.6</td>
<td>8.0</td>
<td>114</td>
<td>—2.4</td>
</tr>
<tr>
<td>3.2</td>
<td>9.0</td>
<td>90</td>
<td>—0.9</td>
<td>9.0</td>
<td>129</td>
<td>—1.2</td>
</tr>
<tr>
<td>6.4</td>
<td>7.0</td>
<td>70</td>
<td>—0.6</td>
<td>8.0</td>
<td>114</td>
<td>—2.1</td>
</tr>
</tbody>
</table>

Table 1

Effect of different doses of aza-dCyd on P388 and L1210 leukemia in CD2F1 mice

The drug was administered i.p. on 5 consecutive days to groups of 6 female mice inoculated with 10^5 cells. Weight changes were registered 6 days after the implantation.
the most effective doses were 0.8 to 1.6 mg/kg, resulting in a 2.5-fold increase of the life-span of treated animals. In L1210 leukemia the longest survival time was observed with doses of 0.2 to 0.4 mg/kg; higher doses were toxic, as evidenced by an increased loss of weight associated with a progressively diminished effect. A comparison of effects of 5-azacytidine and aza-dCyd on AKR mouse leukemia is shown in Table 2. The results indicate that in this system also aza-dCd was toxic at higher doses but was effective at lower doses (0.5 to 1.5 mg/kg). In contrast, increasing doses of 5-azacytidine (up to 4.0 mg/kg) effectively prolonged the life-span of treated mice and were well tolerated. The lethal dose that killed 50% of the female AKR mice was 115 mg/kg for 5-azacytidine and 190 mg/kg for aza-dCyd.

The time schedules of aza-dCyd administered to leukemic AKR mice are shown in Chart 1. In all instances the total dose of the drug was 20 mg/kg, which was divided into 2 doses given on Days 1 and 2, Days 2 and 4 and, finally Days 4 and 5. The last schedule resulted in the longest increase of life-span of the treated mice; however, in combination with a single dose of 5-azacytidine (4 mg/kg) administered on Day 2, this effect was diminished. When the same dose of 5-azacytidine was given on Day 6, i.e., 24 hr after the last dose of aza-dCyd, the survival time was similar.

In our previous work (14) it was observed that 2'-deoxy- cytidine, but not cytidine, partially reversed the antileukemic effect of aza-dCyd. Consequently, we followed the effect of this drug on the incorporation of 2'-deoxycytidine and cytidine into DNA and RNA in livers of leukemic mice. Although the uptake of cytidine into RNA was unaffected by the simultaneous administration of different doses of the analog, the incorporation of 2'-deoxycytidine into DNA was already significantly decreased at the dose of 1 μmole of aza-dCyd (Chart 2). Also, the phosphorylation of 2'-deoxycytidine in the acid-soluble pool was considerably lower in the presence of a 5-fold excess of the inhibitor than it was in the control (Table 3).

These findings led us to follow the inhibitory effect of aza- dCyd on the uptake of 2'-deoxycytidine by cells from leukemic mice in vitro. Chart 3, Curve 1, indicates that the

### Table 2

Comparison of antileukemic effects of 5-azacytidine and aza-dCyd on AKR mouse leukemia

<table>
<thead>
<tr>
<th>Daily dose (mg/kg)</th>
<th>5-Azacytidine</th>
<th>aza-dCyd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival (% survival)</td>
<td>% survival</td>
<td>Survival (% survival)</td>
</tr>
<tr>
<td>0.5</td>
<td>6.5 ± 0.7*</td>
<td>100</td>
</tr>
<tr>
<td>1.0</td>
<td>9.5 ± 1.1</td>
<td>146</td>
</tr>
<tr>
<td>1.5</td>
<td>10.0 ± 1.4</td>
<td>154</td>
</tr>
<tr>
<td>2.4</td>
<td>11.5 ± 0.9</td>
<td>177</td>
</tr>
<tr>
<td>4.0</td>
<td>12.3 ± 1.9</td>
<td>189</td>
</tr>
<tr>
<td></td>
<td>11.4 ± 2.0</td>
<td>176</td>
</tr>
</tbody>
</table>

* Average ± S.E.

<table>
<thead>
<tr>
<th>AzA-dCyd</th>
<th>2'-deoxycytidine, μMole/mouse</th>
<th>Acid-soluble pool (dpm/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7,550</td>
<td>3,690</td>
</tr>
<tr>
<td>0.5</td>
<td>2,167</td>
<td>1,070</td>
</tr>
</tbody>
</table>

J. Vesely and A. Čihák

---

**Chart 1.** Effect of different time schedules of aza-dCyd administration and its combination with 5-azacytidine on the survival time of leukemic AKR mice. Aza-dCyd (●) was administered at a dose of 10 mg/kg i.p. 2 times, as indicated during the period from Days 1 to 5 after s.c. inoculation with 5 × 10^6 leukemic cells. 5-Azacytidine (O), alone or in combination with its 2'-deoxy derivative, was given at a dose of 4 mg/kg i.p. 1 time on Days 2 or Day 6. Each group included 8 female mice (25 g). In all instances the experiments were performed independently in duplicate. The survival times are expressed as percentages (control, 100%) for each experimental group separately and are statistically significant (P < 0.02). The group treated with aza-dCyd on Days 4 and 5 also shows statistical difference when compared to those treated on Days 1 and 2 or Days 2 and 4. Both groups treated with the combination of aza-dCyd and 5-azacytidine are statistically different from the one treated with 5-azacytidine alone (p < 0.02).
The ability of aza-dCyd to block the uptake of 2'-deoxycytidine by cells from leukemic mice and to depress its incorporation into DNA indicated the possible incorporation into DNA of the analog itself. When [3H]aza-dCyd of low-specific radioactivity was added simultaneously with 0.01 μM aza-dCyd (Curve 3) was used; Curve 2, control. The u is expressed as nmole of 2'-deoxycytidine taken up per 1 mg of cell nucleic acids during a 10-mm incubation period.

For experimental details see "Materials and Methods." Bars, S.D.

The ability of aza-dCyd to block the uptake of 2'-deoxycytidine (Fig. 1) in both the lymph nodes and liver was obtained (Table 4). The nuclear label was completely digested with DNase, but it remained unchanged after treatment with cold 5% trichloroacetic acid or with the buffer only.

**DISCUSSION**

The effect of aza-dCyd in P388 and L1210 leukemia is shown in Table 1. It is evident that CD2F1, mice bearing leukemia P388 tolerate higher doses of the drug than do CD2F1, mice bearing L1210 lymphoma. This would apparently indicate that the same drug could have different toxicities and/or metabolic effects in different types of leukemia.

The antileukemic action of aza-dCyd in AKR leukemia in comparison with 5-azacytidine is shown in Table 2. In general the results indicate that the drug has a potent cytostatic action in different types of experimental mouse leukemia.

The dose (4 mg/kg) administered on 5 consecutive days (Table 2) is presumably toxic and does not affect the survival time of treated mice. However, when aza-dCyd was applied at the same total dose (20 mg/kg), divided into 2 portions, and administered 2 times during the 1st 5 days after the inoculation of leukemic AKR cells (Chart 1), the life-span of treated mice was significantly increased. The greatest effect was observed in animals treated on Days 4 and 5 after inoculation during the period of maximal cell growth. The administration of 5-azacytidine before or after aza-dCyd did not exert any beneficial influence (Chart 1).

The administration of 5-azacytidine in AKR leukemia leads to a more favorable response with equally distributed daily doses than it does with an initial high dose followed by small doses (16). The combination therapy of AKR leukemia with 5-azacytidine and aza-dCyd given simultaneously suggests their synergistic effect (14), indicating different modes of action for each compound. Moreover, in the livers of leukemic mice made resistant to 5-azacytidine, uridine kinase activity is considerably decreased, whereas resistance to aza-dCyd is not associated with its impairment, and the sensitivity towards 5-azacytidine is retained (18). During the development of resistance of mouse leukemia to aza-dCyd, the incorporation of 5-azacytidine into infiltrated livers does not change, whereas 5-azacytidine-resistant cells from leukemic mice incorporate the drug to a lesser extent than do the leukemic wild-type cell population (17). It has also been established that, although 5-azacytidine affects the process of liver regeneration in rats and exercises a remarkable modulatory effect on the activity of different eukaryotic enzymes (1, 2), no such action has been observed after the administration of aza-dCyd.

Studies on the distribution of aza-dCyd in mouse tissues show the preferential uptake of the drug into the lymphatic system (4, 13). Twenty-four hr after administration, the concentration of the drug is low in most organs, with the exception of the spleen and thymus. Pretreatment of mice with aza-dCyd results in a strong inhibition of thymidine incorporation into the spleen and thymus that is parallel to the decreased activity of thymidine and thymidylate kinases in the cell-free spleen extracts (4). aza-dCyd does not inter-
Table 4

Labeling of leukemic lymphocytes by [methyl-3H]thymidine or [3H]aza-cCyd in the inguinal lymph nodes and liver of leukemic AKR mice followed by autoradiography

[methyl-3H]Thymidine or [3H]aza-dCyd (200 μCi/0.025 μmol/animal) were given i.p. Two hr later the mice were killed, and their respective tissues were smeared directly on gelatinized slides. Each value is an average from 3 mice; 500 cells ± S.E. were counted in each instance.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Compound administered</th>
<th>Labeled cells (%)</th>
<th>Mean grain count/labelled cell</th>
<th>Synthetic index*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>[methyl-3H]Thymidine</td>
<td>32.5 ± 3.0</td>
<td>12.2 ± 2.5</td>
<td>395</td>
</tr>
<tr>
<td></td>
<td>[3H]aza-dCyd</td>
<td>2.0 ± 0.4</td>
<td>9.0 ± 1.0</td>
<td>18</td>
</tr>
<tr>
<td>Lymph node</td>
<td>[methyl-3H]Thymidine</td>
<td>52.5 ± 4.0</td>
<td>6.1 ± 0.5</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td>[3H]aza-dCyd</td>
<td>10.7 ± 2.5</td>
<td>27.4 ± 6.5</td>
<td>293</td>
</tr>
</tbody>
</table>

* Synthetic index, the number of labeled cells multiplied by the number of grains per average labeled cell.

feres with the metabolism of orotic acid and does not affect RNA synthesis de novo.

The decreased incorporation of 2'-deoxycytidine into DNA from livers of leukemic mice (Chart 2) and its impaired phosphorylation in the acid-soluble pool of cells from leukemic mice in the presence of aza-dCyd (Table 3) suggest the inhibition of DNA synthesis by the drug, which has also been observed in mouse lymphatic tissue labeled with radioactive thymidine (4). On the other hand, they suggest competition with the analog for phosphorylation at the level of deoxycytidine kinase. In mouse leukemic cells in vitro, the equimolar concentration of aza-dCyd depresses the uptake of 2'-deoxycytidine by about 85% (Chart 3). The Lineweaver-Burk plot indicates the competitive nature of the inhibition where $K_i > K_m$ (11).

The results given in Table 4 show that aza-dCyd is incorporated into DNA from tissues of leukemic mice. In leukemic liver the uptake of the drug is considerably less than...
that of thymidine (Table 4). This would explain the failure to obtain significant uptake of [14C]aza-dCyd of low-specific radioactivity into DNA from liver of leukemic mice. The nonspecific incorporation of tritium from [methyl-3H]thymidine into liver RNA has recently been reported (7). This phenomenon does not seem to occur in the case of [3H]aza-dCyd since the labeling of the nuclei of leukemic cells by the tritiated analog has been completely removed with DNase treatment. Moreover, the isolation and chromatography of DNA from livers of leukemic mice labeled with the tritiated drug (Chart 4) clearly indicate the incorporation of the label into DNA molecules.

ACKNOWLEDGMENTS

The authors are indebted to Dr. Bradner of Bristol Laboratories, Division of Bristol-Myers Company, Syracuse, N. Y., for performing the tests with aza-dCyd in P388 and L1210 leukemia reported in Table 1.

REFERENCES

Incorporation of a Potent Antileukemic Agent, 5-Aza-2′-deoxycytidine, into DNA of Cells from Leukemic Mice

J. Veselý and A. Cihák


Updated version Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/37/10/3684

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/37/10/3684. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.