Comparative Biochemical Studies of Adriamycin and Daunomycin in Leukemic Cells

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SUMMARY

Adriamycin (AM) and daunomycin (DM) were compared as inhibitors of nucleic acid and protein biosynthesis in L1210 leukemic cells. In vivo, both antibiotics markedly inhibited, without significant difference, the incorporation of the labeled precursors into DNA and RNA. In vitro, at a concentration of 5 or 10 μg/ml, DM was significantly more effective than AM in inhibiting DNA and RNA synthesis in L1210 leukemic cells. Results of studies with leukemic cells isolated from the blood of patients are the same as those obtained with L1210 cells in vitro.

The reaction catalyzed in vitro by DNA polymerase, isolated from L1210 cells, was equally inhibited by AM and DM, although no effect on the enzyme was seen when the drugs were given in vivo. Studies with L1210 cells, resistant to AM or DM, suggest cross-resistance between the drugs.

INTRODUCTION

AM is a new antitumor antibiotic isolated from cultures of Streptomyces peucetius var. caesius (7). AM differs from its structural analog, DM, in the substitution of a hydroxyl group for a hydrogen atom in the acetyl moiety of DM (Chart 1).

AM has generated interest because its therapeutic index was higher than that of DM in several experimental tumor systems, including solid Sarcoma 180; Ehrlich ascites carcinoma; and ascitic transplantable lymphoma, originally induced by nitrosomethylurea; as well as L1210 and P388 leukemia in mice (7, 13). Clinically, AM has been shown to be effective in the treatment of leukemia and solid tumors (3, 4).

Although AM and DM are closely related in chemical structure, the response to AM in DM-refractory patients suggested that there may be differences in their pharmacological activity. In an attempt to determine whether these differences, as well as those observed in the experimental tumor system, have a biochemical basis, studies were initiated to compare these drugs as inhibitors of nucleic acid and protein biosynthesis in L1210 leukemic cells.

MATERIALS AND METHODS

Chemicals. Thymidine-methyl-3H (6.7 Ci/m mole) and uridine-5-3H (27 Ci/m mole) were purchased from New England Nuclear (Boston, Mass.). L-Valine-3H (8.67 Ci/m mole) and TTP-3H (2.0 to 5.95 Ci/m mole) were purchased from Schwarz BioResearch, Inc. (Orangeburg, N. Y.). Adenosine, deoxyadenosine, thymidine, deoxyctydine, and deoxyguanosine, all as the triphosphates, were purchased from P-L Biochemicals, Inc. (Milwaukee, Wis.). Pyruvic kinase (type II, from rabbit skeletal muscle), calf thymus DNA, and phosphoenolpyruvate were purchased from Sigma Chemical Company (St. Louis, Mo.). Adriamycin and daunomycin were obtained from the Cancer Chemotherapy National Service Center (Bethesda, Md.).

Preparation of Cell Suspensions. Cell suspensions were prepared from L1210 ascitic tumors, grown in DBA/2 female mice, 7 days after an i.p. inoculation of 2 X 10^6 L1210 leukemic cells. The cells were suspended in 10 ml Roswell Park Memorial Institute 1640 medium (10) and kept at 0–4° unless otherwise indicated. Siliconized glassware was used throughout the experiment. The tumor cells were sedimented by centrifugation at 120 X g for 3 min, and the supernatant was removed. After being washed twice with 10 ml of Roswell Park Memorial Institute 1640 medium, the cells were resuspended in the appropriate volume of medium to give a final concentration of 2 to 5 X 10^7 cells/ml.

Human leukocytes were isolated according to the method of Fallon et al. (8). Human blood was collected in a heparinized tube, and the erythrocytes were removed by dextran sedimentation followed by osmotic lysis. Viability was estimated by the ability of cells to exclude 0.025% trypan blue. More than 90% of the cells were viable by this test.

In Vitro Incubation of Cell Suspensions. Aliquots of the cell suspensions were placed in 10- or 25-ml Erlenmeyer flasks, and the appropriate drug was added. Ten μl of stock drug were added to the cell suspension to give the desired concentration of 1 to 10 μg/ml. The control flask contained distilled water in place of the drug. The suspensions were incubated at 37° under a 95% air-5% CO₂ atmosphere in a Dubnoff metabolic shaking water bath. The flasks were shaken just rapidly enough to keep the cells in suspension, and the pH was maintained at 7.2 to 7.3.

Pulse labeling with radioactive precursors was carried out at

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We added 0.5-ml aliquots of the cell suspensions to test tubes that contained 10 µl (1.0 µCi) of the appropriate isotope. The reaction was stopped by chilling the tubes on ice and centrifuging them for 3 min at 200 × g. The supernatant was removed with a Pasteur pipet and the pellet was washed with 1.0 ml of 5% TCA. The samples were resuspended in 5% TCA and collected on Millipore HA filters (0.45 µm) pretwetted with 5% TCA. The filters were washed with 5 ml of 5% TCA and dried for 1 hr at 40°. Each sample was placed in liquid scintillation fluid (2) and counted for 10 min in a Nuclear-Chicago liquid scintillation counter.

**In Vivo Drug Incubation.** The effects of AM and DM in vivo were studied by treating LI210 tumor-bearing mice with 1 i.p. injection of 10 mg/kg of drug. The controls received the same volume of 0.9% NaCl solution. There were at least 3 animals in each group. The animals were killed 2.5 hr after the injection, and the ascitic tumors were removed and pooled. Cell suspensions were prepared by use of the same technique described for the in vitro preparation. The cells were exposed to a 30-min pulse with a radioactive isotope, and incorporation into nucleic acids of protein was measured in the liquid scintillation counter.

**DNA Polymerase, Isolation, and Assay.** The enzyme was isolated from LI210 cells suspended in 4 volumes of a buffered sucrose medium (1). The suspension was homogenized in a Tri-R homogenizer followed by centrifugation at 105,000 × g. The resulting supernatant was used for the DNA polymerase assay.

A modified procedure of Calvin et al. (6) was used to determine enzyme activity. The amount of TTP-3H incorporated into acid-insoluble material was measured. The reaction mixture contained 1 µmole ATP; 20 µmoles potassium phosphate buffer (pH 7.5); 5 µg pyruvate kinase; 100 µg calf thymus DNA (denatured 10 min at 100°); and 150 nmoles each of dATP, dCTP, and dGTP. The reaction was initiated with the addition of 40 nmoles dTTP, which included the labeled precursor (0.5 µCi), and 0.1 ml enzyme preparation (0.5 mg protein). The final reaction volume was brought to 1.0 ml with distilled water. The samples were incubated at 37° for 1 hr. The reaction was stopped by chilling the samples on ice and adding 1 ml of 20% TCA. The samples were then filtered on Millipore filters and washed with 30 ml of 10% TCA. Radioactivity, incorporated into DNA, was determined in the liquid scintillation counter.

**LI210 Cells Resistant to AM and DM.** Resistant sublines of LI210 grown in DBA/2 mice were derived by weekly i.p. injections of increasing concentrations of AM or DM for 4 months and were maintained at 10 mg/kg/week while biochemical studies were being conducted. Experiments were performed by use of both in vivo and in vitro techniques described above. The survival of mice bearing LI210 treated with AM and DM was also studied.

**RESULTS AND DISCUSSION**

The inhibitory effects of AM and DM in vivo on the incorporation of radioactive precursors into DNA, RNA, and protein are compared in Chart 2. Single doses of both drugs, 10 mg/kg, were given 2.5 hr prior to the 30-min pulse with precursors. The incorporation of the labeled precursor into DNA and RNA was markedly inhibited by both compounds,
Table 1

In vivo effect of AM and DM on the incorporation of precursors into nucleic acid of L1210 cells in relation to time

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>% control of thymidine-3 H incorporation into DNA</th>
<th>% control of uridine-3 H incorporation into RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AM</td>
<td>DM</td>
</tr>
<tr>
<td>1</td>
<td>78.0</td>
<td>67.0</td>
</tr>
<tr>
<td>3</td>
<td>67.0</td>
<td>53.5</td>
</tr>
</tbody>
</table>

Table 2

Response of sensitive and resistant L1210 cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>% control of thymidine-3 H incorporation into DNA</th>
<th>% control of uridine-3 H incorporation into RNA</th>
<th>% increase in survival after AM treatment b</th>
<th>MST (days) c</th>
<th>ILS (%) d</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1210</td>
<td>60</td>
<td>56</td>
<td>46</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>L1210/AM</td>
<td>98</td>
<td>97</td>
<td>76</td>
<td>76</td>
<td>9.0</td>
</tr>
<tr>
<td>L1210/DM</td>
<td>94</td>
<td>92</td>
<td>97</td>
<td>99</td>
<td>8</td>
</tr>
</tbody>
</table>

a The results show the effect of 10 mg/kg body weight of the drug given i.p. for 2.5 hr before the animals were killed; the ascites tumor was then removed and the cell suspension was exposed for 0.5 hr to a pulse of the radioactive precursors.
b The results show the effect of 10 mg/kg body weight i.p. of the drug in 2 survival experiments using 5 mice/group.
c MST, median survival time; ILS, increase in median survival time over control.

AM and DM in Leukemic Cells

whereas that into protein was not significantly affected. The inhibitory effect of AM and DM was evident within 1 hr, as shown in Table 1. A similar experiment with mice bearing L1210/AM and L1210/DM showed little or no effect on the incorporation of radioactive precursors into DNA and RNA by either drug when given i.p. (Table 2).

The survival of animals bearing these tumors was also studied. A single i.p. dose of AM (10 mg/kg body weight), given 24 hr after inoculation of the sensitive tumor, increased by 186% the median survival time of mice. There was no increase in the median survival time over the controls of animals bearing the resistant tumors after treatment with AM. Thus, there is an association between the therapeutic effect of AM and its inhibitory effect on nucleic acid biosynthesis. In tumors resistant to AM, this drug did not inhibit the synthesis of nucleic acids. No increase in the survival of mice was found in L1210/DM treated with AM, indicating the cross-resistance of the tumor to AM and DM (Table 2).

In vitro studies were undertaken in an attempt to find differences in the inhibitory effects caused by AM and DM. At a drug concentration of 2 µg/ml, thymidine-3 H incorporation into DNA was inhibited 23% by AM and 48% by DM. Under the same conditions, no inhibition of uridine or valine into RNA and protein incorporation was observed. At a concentration of 5 µg/ml, both AM and DM inhibited the incorporation of labeled precursors into DNA and RNA, and at this concentration DM, but not AM, exerted a significant inhibitory effect on the incorporation of valine into protein. At higher concentrations, DM was significantly more effective than AM in inhibiting DNA and RNA, as well as protein synthesis (Chart 3). The inhibitory effect of AM and DM on the incorporation of thymidine-3 H into DNA and uridine-3 H into RNA was time dependent and occurred as early as 0.5 hr in DM-treated cells. In contrast, the inhibitory effect of AM

![Chart 3](chart3.png)

![Chart 4](chart4.png)
was not significant until 2 hr after exposure to the drug (Charts 4 and 5).

The effects of AM and DM in vitro on resistant cell lines (L1210/AM and L1210/DM), in terms of degree of resistance and cross-resistance, are shown in Table 3. AM, at a concentration of 10 µg/ml, inhibited thymidine-3H incorporation into DNA by 46 and 14% in L1210 and L1210/AM cell lines, respectively. At the same concentration, DM inhibited thymidine-3H incorporation into DNA by 85 and 43% in L1210 and L1210/DM, respectively. Results in Table 3 indicate biochemical cross-resistance of L1210/AM and L1210/DM to AM and DM and are consistent with both in vivo and tumor-inhibitory experiments.

A similar study was carried out with leukemic cells isolated from the blood of 13 children with acute lymphocytic leukemia.

*Chart 5. In vitro effect of various doses of AM and DM on uridine-3H into RNA of L1210 cells as a function of the time of exposure to the drug. In the 0.5-ml incubation mixture, 1 to 2.5 × 10⁷ L1210 cells were exposed to 5 or 10 µg/ml of AM or DM for different time periods prior to the 30-min exposure to 1.0 µCi of the precursors.*

*Table 3
Response of sensitive and resistant L1210 cell lines in terms of degree of resistance and cross-resistance

All determinations are made by the in vitro technique described in "Materials and Methods." Cell counts were the same for sensitive and resistant cell lines. The results show the effect of 10 µg/ml of the drugs.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>% control of thymidine-3H incorporation into DNA</th>
<th>% control of uridine-3H incorporation into RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AM</td>
<td>DM</td>
</tr>
<tr>
<td>L1210</td>
<td>54 ± 11.3¹</td>
<td>15 ± 7.9</td>
</tr>
<tr>
<td>L1210/AM</td>
<td>86 ± 7.6</td>
<td>29 ± 5.6</td>
</tr>
<tr>
<td>L1210/DM</td>
<td>96 ± 14.4</td>
<td>57 ± 4.0</td>
</tr>
</tbody>
</table>

¹ Mean ± S.E.

*Chart 6. In vitro inhibition of the activity of DNA polymerase by AM and DM. DNA polymerase preparations were obtained from L1210 cells and the high-speed supernatant was analyzed for enzyme activity. The average values for 3 experiments are shown.*

*Chart 7. In vivo effect of AM or DM on DNA polymerase. Mice were given AM or DM, 10 mg/kg, in a single i.p. injection. The animals were killed 2.5 hr after the injection, and the ascitic cells were removed and pooled. One-half of this pooled sample was then exposed to 1.0 µCi of thymidine-3H for incorporation studies, and one-half was used for isolation of DNA polymerase. The activity of DNA polymerase was measured in vitro in the presence of added template DNA.*

*Table 4
In vitro effect of AM and DM on thymidine-3H and uridine-3H incorporation into nucleic acid of human leukemic lymphoblasts

Blood samples were obtained from 13 leukemic patients with high WBC and the results are summarized in this table. Leukemic cells were isolated by dextran sedimentation followed by osmolysis to remove RBC. In the 0.5 ml of incubation mixture, 1 to 2.5 × 10⁷ human leukemic cells were exposed to AM or DM, at a concentration of 5 µg/ml, for 2.5 hr prior to the 30-min exposure to 1.0 µCi of the precursors.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Thymidine-3H incorporation into DNA</th>
<th>Uridine-3H incorporation into RNA</th>
<th>Valine-3H incorporation into protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Av. cpm/10⁷ cells</td>
<td>% inhibition</td>
<td>Av. cpm/10⁷ cells</td>
</tr>
<tr>
<td>None</td>
<td>1837 ± 688¹</td>
<td>0</td>
<td>1600 ± 297</td>
</tr>
<tr>
<td>AM</td>
<td>1280 ± 342</td>
<td>35 ± 5.7</td>
<td>1147 ± 255</td>
</tr>
<tr>
<td>DM</td>
<td>681 ± 432</td>
<td>67 ± 5.5</td>
<td>630 ± 190</td>
</tr>
</tbody>
</table>

¹ Mean ± S.E.
leukemia. The differential white blood count of the patients showed more than 60% lymphoblasts in all cases. As shown in Table 4, AM, at a concentration of 5 ìg/ml, inhibited the incorporation of labeled precursors into DNA and RNA, whereas the incorporation of valine into protein was inhibited by only 3%. At the same concentration, DM was significantly more effective than AM in inhibiting the biosynthesis of these macromolecules. These results are essentially similar to those obtained with LI210 cells in vitro.

Results of our studies in vitro are interesting in view of comparative data for AM and DM in a variety of in vivo systems (7, 13). Against 6 different solid or ascitic transplantable rodent neoplasms, AM was the more effective agent. In the present study, the 2 antibiotics were equally effective against L1210 in vivo. However, in the in vitro studies with L1210 and human leukemic cells, DM was the more active agent. Our results are consistent with those of Riehm and Biedler (11), who demonstrated greater activity of DM in cultured Chinese hamster cell lines. The differences between AM and DM, with regard to their antitumor action and effects on nucleic acid biosynthesis, may be due to differences in their metabolism; conceivably, this may involve the conversion of these compounds to a biologically active drug or to differences in their rate of inactivation.

Further studies were carried out to compare the effect of these drugs on DNA polymerase activity. In the in vitro system, both AM and DM produced marked inhibition of DNA polymerase activity (Chart 6). Effects of AM and DM on this enzyme were concentration dependent; 50% inhibition occurred at 7.4 and 5.9 ìg/ml, respectively. Earlier studies (5, 9) have indicated that DM binds to template DNA and thus impairs DNA synthesis in vitro. From these studies, it appears likely that AM acts in a similar manner. When AM and DM were given to tumor-bearing animals 2.5 hr before a pulse of thymidine-3H, the incorporation of this precursor into DNA was significantly impaired. However, the DNA polymerase isolated from these tumors failed to show any inhibition of activity when measured in an in vitro system (Chart 7). Measurements made in the absence of primer DNA showed no significant enzyme activity.

The present study shows that AM and DM inhibit the incorporation of precursors into DNA and RNA. The degree of inhibition is both time and dose dependent, and the effect on thymidine-3H incorporation occurs preferentially at lower doses. The in vitro inhibitory effects of AM and DM on the incorporation of precursors into DNA and RNA was also observed in human lymphoblasts. Thus, at the pharmacological dose, AM and DM appear to act as inhibitors of both DNA and RNA synthesis.

Autoradiographic experiments by Riehm and Biedler suggest that the response of sensitive and resistant cells to DM in a Chinese hamster cell line was determined by a difference in drug uptake. Rusconi and Di Marco (12) also demonstrated a quantitative relationship between the inhibition of uridine-3H incorporation into RNA and uptake of DM. It is therefore possible that there may be a similar difference in their inhibitory effects in both clinical and experimental tumors.

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REFERENCES

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