Antineoplastic Activity of Combinations of
6-Chloropurine and Azaserine

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SUMMARY

Simultaneous administration of 6-chloropurine and azaserine in the therapy of mice bearing the ascites cell forms of Sarcoma 180 or the Ehrlich carcinoma resulted in a potentiation of tumor inhibition as measured by increases in survival time. The 6C3HED and the Mecca ascites lymphosarcomas proved resistant to the combination of chloropurine and azaserine employed. Hepatoma 134 was sensitive to azaserine therapy; addition of chloropurine to this regimen did not elicit a further therapeutic response.

Cytological examination of Sarcoma 180 ascites cells treated with the combination of 6-chloropurine and azaserine revealed the presence of extensive vacuolation, pronounced nucleoli, and both nuclear and cytoplasmic enlargement. These alterations were not present in 6C3HED lymphosarcoma ascites cells treated with the drug combination.

The total packed cell volume was markedly decreased in both Sarcoma 180 and 6C3HED lymphosarcoma ascites cells treated with azaserine. 6-Chloropurine did not decrease the total packed cell volume of either tumor line, while combination of these two agents yielded a decrease similar to that obtained with azaserine in both neoplasms.

Ascites tumor cells possess the potentiality for synthesis of purine nucleotides by two alternate routes. One involves the biogenesis of the nucleotide structure from relatively small molecules (11, 13); the other involves the utilization of the intact purine ring (10). This dual potential for purine nucleotide formation by these neoplasms would imply that a successful chemotherapeutic attack on this area of metabolism would necessitate simultaneous retardation of synthesis through the alternate pathways available to the neoplastic cell.

This concept has been applied by several investigators who have shown that simultaneous administration of azaserine (O-diazoacetyl-L-serine) or DON (6-diazo-5-oxo-L-norleucine), inhibitors of the de novo fabrication of purine nucleotides (1, 6, 8, 9, 12, 13, 21), and analogs of physiological purines resulted in potentiated retardation of tumor growth (3, 4, 7, 17, 20, 23, 25).

One of these purine analogs, 6-chloropurine, has been shown to be an effective inhibitor of a number of transplantable rodent neoplasms (2, 22, 24) and of human acute or chronic leukemia (5, 14). Simultaneous treatment of mice bearing the RC mammary carcinoma with the combination of chloropurine and either azaserine or DON produced a significantly increased number of tumor regressions as compared with controls (25).

In this report, more extensive data are presented on the growth-inhibitory properties of combinations of azaserine and chloropurine with a spectrum of ascites cell neoplasms as test systems.

MATERIALS AND METHODS

Five mouse ascites-cell tumors were employed in these experiments: Sarcoma 180 and the Ehrlich carcinoma in Swiss mice, the 6C3HED lymphosarcoma and Hepatoma 134 in C3H mice, and the Mecca lymphosarcoma in AKR mice.1 Tu-

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minutes in a clinical centrifuge (1600 g), the supernate of peritoneal fluid was decanted, the cells were diluted 1:10 with isotonic saline, and 0.1 ml. of the cell suspension was inoculated into each animal.

Mice were distributed into groups of comparable weight and maintained during experiments on Rockland rat chow pellets and water ad libitum. Drugs dissolved in isotonic saline were injected intraperitoneally; control tumor-bearing animals received injections of the vehicle. Twenty-four hours after tumor implantation, therapy was initiated and continued for 6 consecutive days. Azaserine was used at a dose of 0.2 mg/kg, while the level of chloropurine was varied from 10 to 200 mg/kg. In most cases, these doses were administered twice daily 12 hours apart. Animals were weighed daily during treatments, and the weight change from the onset of therapy was used as an indication of drug toxicity. Although the survival time was routinely used as the criterion of tumor inhibition, in certain cases total packed cell volume and cytological examination were also used.

When survival time was employed, complete regression of tumor growth was established by maintaining surviving animals for over 100 days to allow viable ascites cells to become evident. Tumor-free animals were then autopsied for the presence of solid tumors in the peritoneal cavity; any gross nodules were examined histologically for the presence of malignant cells.

Total packed cell volume was determined by a modification of the method of Sassenrath (19). This was accomplished by sacrificing animals with ether anesthesia on the day following the last drug injection. The animals were weighed, and samples of ascitic fluid from each mouse were withdrawn in capillary tubes which were subsequently heat-sealed. The per cent packed cell volume was obtained after centrifugation in a clinical centrifuge for 10 minutes. Total ascites tumor weight is the difference between the weight of the carcass drained essentially free of ascites cells and that of the tumor-bearing animal. Total packed cell volume was then calculated as the product of the per cent packed cells and the total ascites tumor weight.

For cytological examination, ascitic fluid was withdrawn from mice at various intervals both during and following therapy and was fixed while wet with Bouin’s fixative. Cells were separated from fluid by centrifugation, imbedded in paraffin, and sections of 5–6 μ were cut and stained by the Papanicolaou method.

RESULTS AND DISCUSSION

The effects of chloropurine and azaserine on the survival time of mice bearing Sarcoma 180 ascites cells are presented in Table 1. Throughout these experiments, azaserine was administered at the dosage level which produced essentially complete inhibition of the de novo formation of purine nucleotides in ascites cells (6, 11, 18). Injection of 6-Chloropurine was purchased from Nutritional Biochemicals Corp., Cleveland, Ohio.

<table>
<thead>
<tr>
<th>DAILY DOSAGE (MO/KG)</th>
<th>AVERAGE SURVIVAL (DAYS)</th>
<th>NO. REGRESSIONS</th>
<th>AV. Δ WEIGHT (GM.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azaserine 0</td>
<td>18.0 ± 0.8§</td>
<td>0/20</td>
<td>+7.7</td>
</tr>
<tr>
<td>Chloropurine 0</td>
<td>19.9 ± 1.0</td>
<td>0/20</td>
<td>+1.7</td>
</tr>
<tr>
<td>0.4</td>
<td>18.0 ± 2.2</td>
<td>0/10</td>
<td>+7.1</td>
</tr>
<tr>
<td>0</td>
<td>14.5 ± 0.9</td>
<td>0/10</td>
<td>+8.8</td>
</tr>
<tr>
<td>0.6</td>
<td>12.9 ± 6.9</td>
<td>0/10</td>
<td>+8.8</td>
</tr>
<tr>
<td>0.8</td>
<td>14.8 ± 2.0</td>
<td>0/14</td>
<td>+7.8</td>
</tr>
<tr>
<td>0.12</td>
<td>15.5 ± 7.5</td>
<td>0/10</td>
<td>+6.8</td>
</tr>
<tr>
<td>0.2</td>
<td>13.1 ± 4.3</td>
<td>0/10</td>
<td>+5.5</td>
</tr>
<tr>
<td>0.25</td>
<td>12.6 ± 5.8</td>
<td>0/10</td>
<td>+6.7</td>
</tr>
<tr>
<td>0.4</td>
<td>8.6 ± 0.8</td>
<td>0/10</td>
<td>+7.4</td>
</tr>
<tr>
<td>0.4</td>
<td>20.7 ± 4.2</td>
<td>2/10</td>
<td>+3.1</td>
</tr>
<tr>
<td>0.4</td>
<td>34.2 ± 4.4</td>
<td>5/10</td>
<td>+2.6</td>
</tr>
<tr>
<td>0.4</td>
<td>44.3 ± 4.3</td>
<td>10/15</td>
<td>+1.2</td>
</tr>
<tr>
<td>0.4</td>
<td>46.8 ± 9.9</td>
<td>11/14</td>
<td>+2.3</td>
</tr>
<tr>
<td>0.4</td>
<td>40.2 ± 6.0</td>
<td>6/10</td>
<td>+2.2</td>
</tr>
</tbody>
</table>

* Administered twice daily as two equally divided doses for 6 consecutive days, beginning 24 hours after tumor implantation, with combination treatments given simultaneously.
† Mice surviving over 50 days and tumor-free animals were calculated as 50-day survivors in determination of the average survival time.
‡ Average weight change from onset to termination of drug treatment.
§ Standard deviation of the mean.

and this antibiotic produced a slight prolongation of the survival time of Sarcoma 180 ascites tumor-bearing mice. Chloropurine had no effect on the survival time in daily doses of 20–400 mg/kg body weight; in combination with azaserine, however, it markedly prolonged survival. The most effective combinations employed consisted of daily administration of 0.4 mg/kg of azaserine with 80–80 mg/kg of chloropurine. When this dosage schedule was employed, 75 per cent of the tumors regressed.

Clarke et al. (4) have reported that subcutaneous implants of Sarcoma 180 were sensitive to combined therapy with azaserine and chloropurine. However, at the effective dose levels, delayed mortality occurred. In all experiments with Sarcoma
180 described in this manuscript, no indication of
drug toxicity was observed with the drug combina-
tions employed. This difference in toxicity is a
function of the lower dose levels which can be used
in the ascites test system.

Table 2 illustrates the effect of simultaneous and
alternate drug therapy on the survival time of Sarcoma 180 ascites tumor-bearing mice. Animals
on alternate therapy received azaserine at approximately 8:00 A.M. and chloropurine at 8:00 P.M.
This drug schedule did not prove to have any ad-

tage over the azaserine treatment, since these mice had essentially the same average survival
time as animals receiving alternate drug therapy.

The data obtained with other ascites-cell neo-
plasms are recorded in Table 3. Azaserine was
combined with chloropurine at the level found to
be optimal for the inhibition of Sarcoma 180 ascites
cells. Injections of azaserine caused a slight
prolongation of survival time, while chloropurine
proved to have no effect on the Ehrlich ascites car-
cinoma. With this tumor, regressions occurred in
40 per cent of the animals when daily doses of 80
mg/kg of chloropurine with 0.4 mg/kg of azaserine
were given. Administration of a higher dose of
chloropurine with azaserine was found to be toxic.
The 6C3HED and the Mecca ascites-cell lympho-
sarcomas were found to be relatively resistant to
the combination employed. Hepatoma 184 was
sensitive to azaserine therapy, which produced a
doubling of the survival time. Chloropurine ad-
ministration at 80 mg/kg did not prolong the sur-
vival time of mice bearing this neoplasm nor did it
appear to potentiate the inhibition by azaserine.

Cytological examination of drug-treated ascites
cells was carried out during and following the ther-
apy period. Figure 1 illustrates a section of un-
treated Sarcoma 180 ascites cells which were ob-
tained from an animal bearing a 7-day tumor
growth. Examination of Sarcoma 180 ascites cells
on the day following a course of azaserine therapy
revealed extensive enlargement of both the nucleus
and the cytoplasm with the presence of prominent
nucleoli and large vacuoles (Fig. 2). Enlarged cells
were still present 3 days after termination of drug
injections. Similar findings for azaserine-treated
Ehrlich ascites cells have been reported (6). The
giant cells present in the population of chloro-
purine-treated Sarcoma 180 cells on the day fol-

dowing therapy are depicted in Figure 3, and the
effect of the combination of azaserine and chloro-
purine is shown in Figure 4. As in the other drug-
treated groups, nuclear and cytoplasmic enlarge-
ment with extensive vacuolation and prominent
nucleoli was seen both during and following ther-
apy.

The cytological changes observed in the chloro-
purine-treated cells were in accord with biochemi-

cal data which demonstrated inhibition by chloro-
purine of glycine-2-C$^{14}$ utilization for nucleic acid
guanine synthesis in this neoplasm (16). The in-
ability of chloropurine to prolong the survival time
of Sarcoma 180 tumor-bearing mice would suggest
that the biochemical and cytological alterations
are short-lived. Examination of chloropurine-
treated Sarcoma 180 cells 3 days after termination
of therapy supported this view, since, although en-
larged forms were present, the population ap-
peared to contain many viable tumor cells (Figs. 5,
6).

Examination of 6C3HED ascites cells harvested
from mice on the day following completion of a 6-
day course of therapy with either azaserine, chloro-
purine, or the combination of these two agents did
not reveal the presence of giant forms or cells
showing extensive vacuolation. These findings cor-
roborated the survival data, which indicated that
this neoplasm was relatively resistant to these
drugs, and were consistent with biochemical find-
ings which indicated that chloropurine did not al-
ter the rate of incorporation of glycine-2-C$^{14}$ into
nucleic acid guanine.

Since chloropurine produced cytological changes
in Sarcoma 180 ascites cells without prolonging the
survival time of mice bearing this neoplasm, it was
of interest to attempt to estimate the chemothera-
pic efficacy of this agent by another criterion.

These results, which are recorded in terms of total
packed cell volume, are presented in Table 4. De-
termination of total packed cell volume for both
the Sarcoma 180 and the 6C3HED lymphosar-
comas was carried out on the day following the ter-
mination of the drug injections. Treatment of Sar-
coma 180 ascites tumor-bearing mice with azas-
erine afforded a 74 per cent reduction in the total
packed cell volume, while chloropurine did not re-
duce the total packed cell volume. The combina-
tion of azaserine with chloropurine appeared to be
equivalent to the azaserine-treated group. Inter-
pretation of results obtained with this criterion of
tumor inhibition was limited in these instances,
since it is evident that this measurement is insensi-
tive to changes in cell size.

Results with the 6C3HED lymphosarcoma indi-

4A. C. Sartorelli and B. A. Booth, unpublished observa-
tions.
### TABLE 2

**EFFECTS OF SIMULTANEOUS AND ALTERNATE DRUG THERAPY ON SURVIVAL TIME OF SARCOMA 180 ASCITES TUMOR-BEARING MICE**

<table>
<thead>
<tr>
<th>Daily Dosage* (mg/kg)</th>
<th>Therapy†</th>
<th>Average Survival (Days)</th>
<th>No. Regressions‡</th>
<th>Av. Δ Weight§ (gm.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azaserine</td>
<td>Chloropurine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>—</td>
<td>14.6±2.0‡</td>
<td>0/10</td>
</tr>
<tr>
<td>0.2</td>
<td>0</td>
<td>—</td>
<td>20.4±1.1</td>
<td>0/10</td>
</tr>
<tr>
<td>0</td>
<td>40</td>
<td>—</td>
<td>17.2±1.8</td>
<td>0/10</td>
</tr>
<tr>
<td>0.2</td>
<td>40</td>
<td>Alternate</td>
<td>20.8±2.4</td>
<td>0/15</td>
</tr>
<tr>
<td>0.2</td>
<td>40</td>
<td>Simultaneous</td>
<td>38.3±3.6</td>
<td>6/15</td>
</tr>
</tbody>
</table>

* Administered once daily for 6 consecutive days, beginning 24 hours after tumor implantation.
† Alternate therapy consisted of 0.2 mg/kg of azaserine injected at approximately 8:00 A.M. and 40 mg/kg of chloropurine at 8:00 P.M.; simultaneous therapy consisted of administration of both agents at 8:00 A.M.
‡ Mice surviving over 50 days and tumor-free animals were calculated as 50-day survivors in determination of the average survival time.
§ Average weight change from onset to termination of drug treatment.
# Standard deviation of the mean.

### TABLE 3

**EFFECTS OF DRUG THERAPY ON THE SURVIVAL TIME OF ASCITES TUMOR-BEARING MICE**

<table>
<thead>
<tr>
<th>Neoplasms</th>
<th>Daily Dosage* (mg/kg)</th>
<th>Average Survival (Days)</th>
<th>No. Regressions†</th>
<th>Av. Δ Weight‡ (gm.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ehrlich carcinoma</td>
<td>Azaserine</td>
<td>Chloropurine</td>
<td>11.1±1.1§</td>
<td>0/15</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>—</td>
<td>18.5±1.1</td>
<td>0/15</td>
</tr>
<tr>
<td>0.4</td>
<td>0</td>
<td>—</td>
<td>9.1±1.0</td>
<td>0/15</td>
</tr>
<tr>
<td>0</td>
<td>150</td>
<td>—</td>
<td>8.7±0.6</td>
<td>0/10</td>
</tr>
<tr>
<td>0</td>
<td>200</td>
<td>—</td>
<td>11.3±2.2</td>
<td>0/10</td>
</tr>
<tr>
<td>0.4</td>
<td>80</td>
<td>—</td>
<td>42.7±3.4</td>
<td>6/15</td>
</tr>
<tr>
<td>0.4</td>
<td>150</td>
<td>—</td>
<td>8.0±1.2</td>
<td>0/5</td>
</tr>
<tr>
<td>6CSHED lymphosarcoma</td>
<td>0</td>
<td>0</td>
<td>11.7±0.7</td>
<td>0/15</td>
</tr>
<tr>
<td>0.4</td>
<td>0</td>
<td>—</td>
<td>14.5±0.7</td>
<td>0/15</td>
</tr>
<tr>
<td>0</td>
<td>80</td>
<td>—</td>
<td>11.7±0.3</td>
<td>0/15</td>
</tr>
<tr>
<td>0.4</td>
<td>80</td>
<td>—</td>
<td>13.4±0.3</td>
<td>0/14</td>
</tr>
<tr>
<td>Mecca lymphosarcoma</td>
<td>0</td>
<td>0</td>
<td>8.1±0.1</td>
<td>0/20</td>
</tr>
<tr>
<td>0.4</td>
<td>0</td>
<td>—</td>
<td>9.3±0.2</td>
<td>0/20</td>
</tr>
<tr>
<td>0</td>
<td>80</td>
<td>—</td>
<td>8.0±0.2</td>
<td>0/20</td>
</tr>
<tr>
<td>0.4</td>
<td>80</td>
<td>—</td>
<td>10.0±0.2</td>
<td>0/20</td>
</tr>
<tr>
<td>Hepatoma 134</td>
<td>0</td>
<td>0</td>
<td>11.8±1.4</td>
<td>0/20</td>
</tr>
<tr>
<td>0.4</td>
<td>0</td>
<td>—</td>
<td>24.4±2.0</td>
<td>1/20</td>
</tr>
<tr>
<td>0</td>
<td>80</td>
<td>—</td>
<td>9.2±1.3</td>
<td>0/20</td>
</tr>
<tr>
<td>0.4</td>
<td>80</td>
<td>—</td>
<td>21.0±2.0</td>
<td>0/20</td>
</tr>
</tbody>
</table>

* Administered twice daily as two equally divided doses for 6 consecutive days, beginning 24 hours after tumor implantation, with combination treatments given simultaneously.
† Mice surviving over 50 days and tumor-free animals were calculated as 50-day survivors in determination of the average survival time.
‡ Average weight change from onset to termination of drug treatment.
§ Standard deviation of the mean.
cated that this tumor was sensitive to azaserine, which produced a 74 per cent reduction in the total packed cell volume. Cells from mice injected with chloropurine yielded a total packed cell volume similar to the value for untreated animals. Combination of chloropurine and azaserine produced essentially the same reduction as was found in the azaserine-treated group. Although inability of the drug combination to prolong the survival time of 6C3HED ascites tumor-bearing mice appears to be related to the absence of a sensitive enzyme along the pathway leading to the synthesis of nucleic acid guanine, the results may be partially due to the rapid invasion of host tissues by the ascites lymphomas (15).

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REFERENCES

13. MOORE, E. C., and LEPAGE, G. A. In Vivo Sensitivity of Hepatoma 134 was kindly supplied by Dr. Morris Belkin of the National Cancer Institute; the Ehrlich carcinoma, 6C3HED lymphosarcoma, and Sarcoma 180 by Dr. G. A. LePage of the Stanford Research Institute; and the Methylcholanthrene lymphosarcoma by Dr. Kanematsu Sugiura of the Sloan-Kettering Institute.

We are indebted to Dr. John R. Rice, Research Division, Parke, Davis & Co., for a generous gift of azaserine.

**TABLE 4**

<table>
<thead>
<tr>
<th>Neoplasm</th>
<th>Treatment*</th>
<th>No. mice</th>
<th>Average total packed cell volume†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarcoma 180</td>
<td>Control</td>
<td>8</td>
<td>1.90 ± 0.13†</td>
</tr>
<tr>
<td>Azaserine</td>
<td>9</td>
<td>0.50 ± 0.20†</td>
<td></td>
</tr>
<tr>
<td>Chloropurine</td>
<td>9</td>
<td>2.44 ± 0.26†</td>
<td></td>
</tr>
<tr>
<td>Azaserine+</td>
<td>chloropurine</td>
<td>9</td>
<td>0.65 ± 0.14†</td>
</tr>
<tr>
<td>6C3HED lymphosarcoma</td>
<td>Control</td>
<td>10</td>
<td>0.31 ± 0.05§</td>
</tr>
<tr>
<td>Azaserine</td>
<td>8</td>
<td>0.05 ± 0.05§</td>
<td></td>
</tr>
<tr>
<td>Chloropurine</td>
<td>12</td>
<td>0.94 ± 0.05§</td>
<td></td>
</tr>
<tr>
<td>Azaserine+</td>
<td>chloropurine</td>
<td>10</td>
<td>0.06 ± 0.02§</td>
</tr>
</tbody>
</table>

* Azaserine was administered at a level of 0.4 mg/kg and chloropurine at 80 mg/kg injected twice daily as equally divided doses for 6 consecutive days, beginning 24 hours after tumor implantation, with combination treatments given simultaneously. Controls were tumor-bearing animals receiving injections of isotonic saline.

† Standard deviation of the mean.

**Fig. 1.—** Sarcoma 180 ascites tumor cells harvested from an untreated mouse 7 days after tumor implantation. Prepared by the Papanicolaou method. ×896.

**Fig. 2.—** Sarcoma 180 ascites tumor cells 1 day following treatment with six daily divided doses of 0.4 mg/kg of azaserine. The section illustrates the nuclear enlargement, cytoplasmic enlargement, increased vacuolation, and pronounced nucleoli which result from this therapy. ×896.

**Fig. 3.—** Sarcoma 180 ascites tumor cells 1 day following treatment with six daily divided doses of 80 mg/kg of 6-chloropurine. ×896.

**Fig. 4.—** Sarcoma 180 ascites tumor cells 1 day following treatment with six daily divided doses of 0.4 mg/kg of azaserine and 80 mg/kg of 6-chloropurine. ×896.
Fig. 5.—Sarcoma 180 ascites tumor cells harvested from an untreated mouse 9 days after tumor implantation. ×896.

Fig. 6.—Sarcoma 180 ascites tumor cells 3 days following treatment with six daily divided doses of 80 mg/kg of 6-chloropurine. ×896.


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