Chemotherapeutic, Carcinogenic, and Cell-regulatory Effects of Triazenes

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SUMMARY

Chemotherapeutic, carcinogenic, and cell-regulatory effects of 1-phenyl-3-monomethyltriazene (PMT), 1-phenyl-3,3-dimethyltriazene, and 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide have been determined. The maximum increase (%) in median survival time of mice bearing the L1210 leukemia, after i.p. treatment with PMT, was 38 for a single dose and 50 for 6 daily injections. The effects of PDT and DIC were similar.

PMT (45 mg/kg in sesame oil) injected s.c. in 4 weekly doses caused death due to local tumors in 9 of 11 AKR/J and in all 9 C57BL/6J mice. Applied to the skin, PMT in C8H8 produced papillomas in both strains to the same extent.

Treatment for several transfers with PMT (30 mg/kg in 6 daily i.p. doses) resulted in the decrease of oncogenic potential in the following neoplasms: L1210, P815, L5178Y, and L5178Y/CA55.

Following exposure of L1210 cells to 1 to 4 mM solutions of the triazenes at 3°, only PMT was cytotoxic.

INTRODUCTION

Recently, it was reported that treatment of L1210 with DIC2 (1, 8), PDT, and PMT (8) (Chart 1) for several transplant generations increased markedly the MST of the untreated control leukemic animals. In subsequent untreated transplants, these sublines either did not take or retained reduced growth potential. We suggested that this change in growth potential is caused by an action of the carbonium ion that is formed on decomposition of monomethyltriazines (6, 10).

According to the prevailing present concept, most if not all chemical carcinogens are in their ultimate carcinogenic form, electrophilic reactants, similar to the alkylating agents used in cancer chemotherapy (4). The purpose of the present study is to demonstrate chemotherapeutic, carcinogenic, and cell-regulatory properties of the 3 mentioned triazenes, especially of PMT. The definition of cell regulation in this paper is cell change following treatment with drugs such as the triazenes.

MATERIALS AND METHODS

The following mouse strains were used: C57BL/6 × DBA2 F1 (hereafter called BD2F1), 6 to 10 weeks old (A. R. Schmidt Co., Madison, Wis.), AKR/J, and C57BL/6J (The Jackson Laboratory, Bar Harbor, Maine). All groups consisted of 5 or 10 male mice. The mouse neoplasms studied were the parental line L1210 and sublines of L1210 resistant to methotrexate and 6-methylmercaptopurine riboside; L5178Y and its cytosine arabinoside-resistant subline L5178Y/CA55; and mast cell neoplasm P815 and its cytosine arabinoside-resistant subline.

The antitumor effect of the 3 triazenes was studied with L1210. One million cells were implanted i.p. Treatment was started 1 day later and administered either i.p., s.c., or p.o., as a single injection or in 6 injections, 1 per day. The single dose of each drug used was lethal to 10% of the animals. These doses were determined by plotting dose-mortality data, which were obtained in normal male BD2F1 mice, on probability paper. The doses for 6 daily treatments caused less than 50% mortality in normal mice; a doubling of the doses caused death in most of the mice. DIC was suspended in 0.5% carboxymethylcellulose in dark bottles shortly before use. PDT and PMT were each dissolved in sesame oil and stored frozen at about –20°. DIC was supplied by the Drug Development Branch, Drug Research and Development, Chemotherapy, National Cancer Institute, Bethesda, Md. PDT was obtained from Merck and Co., Rahway, N. J. PMT was synthesized by us (8).

To induce tumors, 1 dose of PMT, 45 mg/kg, dissolved in...
0.9 ml sesame oil was administered s.c. weekly for 4 weeks in the right axillary region of AKR/J and C57BL/6J mice. PMT is a direct alkylating agent and an acute irritant. Therefore, a large volume was used to decrease its concentration and to prevent it from being ulcerated out. Sesame oil was selected because we used it in previous studies (7). The mice were checked once a week for 7 months, after which the survivors were killed. Tumors were histologically examined. Latent period is defined as the time between the 1st injection of the carcinogen and appearance of tumor; survival time is defined as the time between injection and death due to tumor.

For the production of papillomas, multiple applications of 1 drop of 1, 2, or 4% of PMT in benzene were made, twice a week for 8 weeks, to the skin on the middle of the back of AKR/J and C57BL/6J mice. PMT was applied with a medicine dropper which delivered 45 drops/ml, i.e., 222 μg of PMT per drop of the 1% solution. The controls received the corresponding amount of benzene. The mice were checked for the development of papillomas once a week for 20 weeks, after which the survivors were killed. Only lesions that were sharply defined, raised, and larger than 2 mm were considered as papillomas. Regressions have not been tabulated; the regression rate was similar in all experimental groups. The relatively short observation time of 20 weeks was chosen because of the short life-span of the AKR/J mice due to spontaneous leukemia and because of the severe ulceration caused by PMT at the site of application in both strains.

The development of PMT-treated tumor lines consisted of treatment with PMT, 30 or 15 mg/kg, in 5 or 6 daily i.p. injections for 15 transplant generations. In each transplant generation, 1 million ascites cells obtained from the treated mice were implanted i.p. into 2 groups of mice, untreated and treated.

Further experimental details are described in the individual tables.

RESULTS

The results obtained with the 10% lethal doses of the 3 triazines in L1210 differed little (Table 1). After 1 i.p. administration, the maximum increase (%) in MST was 38 for both PMT and DIC. The MST's were in most cases greater after 6 daily injections than after treatment with single doses. DIC was slightly superior to PMT and PDT; it produced an increase of 63% in the MST of the i.p.-treated mice.

Table 2 shows the carcinogenic potency of PMT. All C57BL/6J and 9 of 11 AKR/J mice died of local tumor formation within 6 months after the 1st injection of PMT. One control mouse also developed a tumor at the site of injection of the sesame oil. Histologically, all tumors were sarcomas.

Multiple applications of PMT, in benzene, to the skin of the backs of AKR/J and C57BL/6J mice resulted in papilloma formation to about the same degree in both strains (Table 3). Three AKR/J and 6 C57BL/6J mice died because of the severe ulcerations caused by PMT.

The cytocidal effect of in vitro treatment with PMT and the other 2 triazines on L1210 cells has been examined (Table 4). Following exposure of L1210 cells to 1 to 4 mM solutions at 3° for 1 hr, DIC and PDT had no effect, but PMT was toxic at all concentrations. After incubation for 30 min at 37°, PMT was completely cytocidal and PDT was slightly to moderately cytocidal.

The effect of PMT in the treated groups of the 1st generation of the various leukemia lines represents the antineoplastic activity of PMT (Tables 5 and 6). The MST's were 11 days for both the L1210 line resistant to methotrexate and for the mast cell tumor P815, versus 8 days for the controls. The MST's of the treated groups of L5178Y and its subline L5178Y/CA55 were slightly prolonged. After several treatment generations, the MST's of the untreated control mice did...
not take in the 11th generation, and P815 did not take in the 15th generation. L5178Y and L5178Y/CA55 showed only a temporary increase in MST of the untreated controls. Four additional sublines that were lost due to treatment with PMT, 30 mg/kg, for 15 generations were: a P815, a P815/ara-C, an L1210 line resistant to 6-methylmercaptopurine riboside, and an L5178Y/CA55 line.

The untreated controls of all 3 L1210 sublines that were treated with PMT, 15 mg/kg, had MST's greater than 30 days at Generation 10 (Table 6). During subsequent untreated generations of those lines, the MST's gradually decreased, and, by Generation 37, they were similar to that of the untreated parent L1210 line. An additional series of treated and untreated generations showed again the rise and gradual decrease of the MST's.

The MST's of mice given injections of various proportions of L1210 cells and PMT-altered L1210 cells (L1210/PMT) are shown in Table 7. From several mice of the group that received 1 L1210 and 1 million L1210/PMT cells, ascites cells were pooled and transplanted into normal BD2F1 mice. After 2 transfers, the MST was as low as that of mice bearing the L1210 leukemia.

### Table 3

**Papilloma production and skin ulceration with PMT**

<table>
<thead>
<tr>
<th>Total dose (mg)</th>
<th>No. of mice Initially</th>
<th>At 20 wk</th>
<th>No. of mice with ulceration at site of application</th>
<th>Mice with papillomas at 20 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>10</td>
<td>10</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>7.1</td>
<td>10</td>
<td>8</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>14.2</td>
<td>10</td>
<td>9</td>
<td>10</td>
<td>5</td>
</tr>
</tbody>
</table>

**AKR/J male**

3.5
7.1
14.2

**CS7BL/6J male**

3.5
7.1
14.2

### DISCUSSION

From the antileukemic effects obtained with the 3 triazenes, it is clear that although the tolerated dose range is lower for PMT and PDT they are only slightly less effective than DIC in increasing the survival time of leukemic mice. Similarly, Lin et al. (3) found 6 derivatives of PDT, and DIC were equally active against L1210. The pyrazole analog of DIC likewise displays an antileukemic property (5, 9). Therefore, it appears that the imidazole ring, the phenyl ring, and the pyrazole ring are all equally effective carriers of the triazene group. Probably, the triazenes act as alkylating agents through the in vivo generation of carbonium ions (6).

Carcinogenicity has been clearly demonstrated for all 3 triazenes. All rats fed DIC developed mammary carcinomas (11). The p.o. and s.c. administration of PDT to rats

### Table 4

**The transplantability of L1210 cells after in vitro exposure to PMT, PDT, and DIC**

L1210 cells, 10⁷/ml, were incubated in Earle's solution. PMT and PDT were dissolved in dimethyl sulfoxide and DIC was dissolved in 0.1 N HCl, and each was added as 0.2- to 0.8-ml portions to 10 ml of the incubation mixture. Incubation time for 37° was 30 min, and for 3° was 60 min. Groups of 5 BD2F1 mice were given i.p. injections of about 1 million cells.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>PMT</th>
<th>PDT</th>
<th>DIC</th>
<th>Dimethyl sulfoxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°</td>
<td>&gt;30</td>
<td>9</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>23</td>
<td>7</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>8</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>&gt;30</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>

Carcinogenecity has been clearly demonstrated for all 3 triazenes. All rats fed DIC developed mammary carcinomas (11). The p.o. and s.c. administration of PDT to rats...
produced tumors mainly in the brain and peripheral nervous system; very few local sarcomas were induced by s.c. treatment (2, 6). PMT also proved to be a powerful carcinogen in the rat. On p.o. administration, all animals died with local carcinomas in the esophagus and forestomach. The s.c. injections produced predominantly local carcinomas at the site of injection. Our results show that PMT is also a carcinogen in the mouse. Application to the skin produced papillomas uniformly, and s.c. application produced local tumors at the site of injection in 18 of 20 mice in a relatively short time. These results tend to agree with the mutagenic activity reported by Vogel et al. (12).

The dimethyltriazenes are metabolically demethylated to the monomethyltriazenes. These are very unstable and, on decomposition, yield the active agent, the carbonium ion (6, 10). As was to be expected, in vitro treatment with only PMT was cytotoxic for L1210 cells, as determined by bioassay into normal mice. The slight to moderate effect of PDT at 37° may have been due in part to its demethylation by the L1210 cells to PMT. Because the monomethyl compound appears to be the direct alkylating agent and the proximate carcinogen, we used PMT for further attempts to elucidate the mechanism of cell regulatory properties of these triazenes. Whereas a treatment with 30 mg/kg for 6 days during 10 transplant generations resulted in the loss of L1210 (8), of the mast cell neoplasm P815, and of resistant lines derived from them, it caused only a temporary increase in the MST of the controls of L5178Y and L5178Y/CA55.

Treatment of L1210 with the lower dose of 15 mg/kg increased the MST of the untreated controls. During untreated transfer generations, the lines slowly became more oncogenic, but only after 37 generations did they approach the level of the parental L1210. In contrast, only 2 generations were necessary to reach the MST of the L1210 line, if only about 1 L1210 cell was added to 1 million
Table 7

<table>
<thead>
<tr>
<th>No. of cells</th>
<th>L1210</th>
<th>L1210/PMT</th>
<th>MST (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^6</td>
<td>0</td>
<td>10^6</td>
<td>8</td>
</tr>
<tr>
<td>10^7</td>
<td>10^7</td>
<td>10^7</td>
<td>9</td>
</tr>
<tr>
<td>10^8</td>
<td>10^8</td>
<td>10^8</td>
<td>10</td>
</tr>
<tr>
<td>10^9</td>
<td>10^9</td>
<td>10^8</td>
<td>11</td>
</tr>
<tr>
<td>10^10</td>
<td>10^10</td>
<td>10^9</td>
<td>12</td>
</tr>
<tr>
<td>10^11</td>
<td>10^11</td>
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<td>14</td>
</tr>
<tr>
<td>10^12</td>
<td>10^12</td>
<td>10^11</td>
<td>&gt;30</td>
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<td>10^13</td>
<td>10^13</td>
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</tr>
<tr>
<td>10^14</td>
<td>0</td>
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<td></td>
</tr>
<tr>
<td>10^15</td>
<td>0</td>
<td>10^14</td>
<td></td>
</tr>
</tbody>
</table>

*a* Untreated transfer Generation 2 of an L1210 line treated for 15 generations with PMT, 30 mg/kg, for 6 days.

*b* This group is donor for Generation 2.

PMT-altered L1210 cells. These experiments seem to rule out the possibility that selection was the cause for the increase in survival times of the triazene-treated lines. The increased generation time of the treated cells (8) is probably partly due to intrinsic, biochemically adaptive changes in the cells.

The current findings demonstrate cytotoxic, carcinogenic, and cell-regulatory (cell change) potencies of the 3 triazenes. Cytotoxicity and carcinogenicity always commanded major interest in cancer research, but cell regulation appears to have been neglected. Understanding the factors that make a tumor cell less malignant might well be the way to a complete control of cancer.

REFERENCES


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