Experimental Studies on the Antitumor Effect of Ethidium Bromide and Related Substances

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SUMMARY

The antileukemic activity of ethidium bromide, an inhibitor of RNA-dependent DNA polymerase activity, and of 14 related agents newly synthesized was tested in experimental tumor systems. Ethidium bromide caused up to 200% increased life-span in mice with 6C3HED-OG and 83% increased life-span in mice with L5178Y. No substantial percentage of increased life-span was noted in rodents with L1210, 6C3HED-RG, EL4, RADA1, and Walker carcinosarcoma 256. Of the 14 related substances tested, PD-MY-001 and PD-MY-003 also increased the survival time of mice with 6C3HED-OG up to 200%. Ethidium bromide and these two newly synthesized substances are considered to be antitumor agents with a unique mechanism of action.

INTRODUCTION

The RNA-containing murine and avian oncogenic viruses have both RNA-dependent and DNA-dependent DNA polymerase activities (1, 3, 10, 11). The RNA-dependent DNA polymerase activity has now been discovered in Visna (8, 9) and “foamy” viruses (9) and in leukemic cells from patients with acute lymphoblastic leukemia (2). It has been shown that this RNA-dependent DNA polymerase activity is suppressed by 4-Af-demethylrifampicin and 4-/V-benzyldemethylrifampicin, derivatives of rifampicin (4). Hirsclunan (5) reported that EB2 had stronger action than did rifampicin derivatives in suppressing the DNA polymerase activity of Rauscher or Moloney murine leukemia viruses. Discovery of inhibitors of nucleic acid synthetase of RNA-containing tumor viruses would be useful not only for analysis of the mechanism of virus replication, but also for determination of chemotherapeutic implications. The antitumor effect of EB, which suppressed this polymerase activity, and of 14 of its newly synthesized related substances was studied in experimental tumor system.

1 The present study was supported by the Grant-in-Aid for Scientific Research from the Ministry of Education of Japan.

2 The abbreviations used are: EB, ethidium bromide; ILS, increased life-span.

Received December 10, 1973; accepted June 3, 1974.

MATERIALS AND METHODS

In Vivo Experimental System

Experimental Animals. Test animals included mouse strains CBA, C57BL X DBA/2 F1, C57BL, DDY, SMA (pure strain animals supplied by the Nagoya University School of Medicine, Nagoya, Japan), and A/J (The Jackson Laboratory, Bar Harbor, Maine) of both sexes, 2 to 3 months old, and weighing about 25 g. Two-month-old adult Sprague-Dawley rats (Chubu Kagaku Shizai, Nagoya, Japan) weighing about 60 g were also used.

Experimental Tumors. 6C3HED Gardner lymphosarcoma OG (l-asparaginase-sensitive strain), 6C3HED Gardner lymphosarcoma RG (l-asparaginase-resistant strain), L1210 leukemia, L5178Y leukemia, EL4 leukemia, Friend virus leukemia, RADA1 leukemia, and Walker carcinosarcoma 256 were used.

Chemical Agents. Chemical agents studied were EB and 14 related substances, the structures of which are shown in Chart 1. The EB (2,7-diamino-10-ethyl-9-phenylphenanthridinium bromide) was purchased from Sigma Chemical Co., St. Louis, Mo. The related substances were synthesized and supplied by Kyowa Hakko Kogyo Ltd., Tokyo, Japan. EB was dissolved in 0.85% NaCl solution and the related substances were suspended in 0.5% carboxymethylcellulose and injected i.p. into mice or rats.

Examination of Antitumor Activity. Mice or rats were given tumor cells suspended in 0.85% NaCl solution either i.p. or s.c. The cell suspension was such that each mouse usually received 1 X 10^6 tumor cells, except mice bearing L1210 in which 1 X 10^5 cells were inoculated, and each rat usually received approximately 1 cu mm of solid tumor tissue. Effectiveness of the treatments was determined by the percentage of increase in mean survival time of the treated groups over the control group. A 25% increase was considered the minimum response indicative of consequential antileukemic activity.

In Vitro Drug Sensitivity Test

The effect of EB and related substances on DNA and RNA synthesis was examined in vitro using various experimental leukemias.

In the experiments using the tumors 6C3HED OG, 6C3HED RG, L1210, EL4, and L5178Y, 1 X 10^6 (0.1 ml) tumor cells
were added to 0.8 ml of Eagle's minimum essential medium (Institute of Microbiological Research, Osaka University, Osaka, Japan) with the addition of 15% fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.). These mixtures were inactivated at 56°C for 30 min and dialyzed in test tubes for incubation. To each of these test tubes, 0.1 ml of various concentrations of the chemical solution was added and incubated at 37°C for 2 hr. In the control group, the same volume of 0.85% NaCl solution was added in place of the chemical solution. Further, 0.5 μCi of uridine-5-3H (specific activity, 6.0 Ci/mmole; Daiichi Pure Chemicals Co., Tokyo, Japan) and 0.05 μCi of thymidine-2-14C (specific activity, 50.0 mCi/mmole; Daiichi Pure Chemicals Co.), adjusted to 0.1 ml, were added and incubated for 4 hr. The mixture was filtered through a Millipore filter (HAWP 02500, 25 ea, HA 0.45 μm, white plan 25 mm, Millipore Co., Bedford, Mass.), cold 5% trichloroacetic acid (Katayama Chemical Works, Tokyo, Japan) was poured over the cells on the filter, and the filter was transferred to the liquid scintillator vial (Beckman Instruments, Inc., Fullerton, Calif.). After drying at room temperature, 10 ml of a scintillator solution of 500 ml toluene (Katayama Chemical Works, Osaka, Japan) containing 2.3 g of PPO (Wako Pure Chemicals Co., Osaka, Japan) and 0.05 g of POPOP (Wako Pure Chemicals Co., Osaka, Japan) were added and radioactivity was measured by the liquid scintillation counter, Aloka LSC-502 (Japan Radiation and Medical Electronics, Inc., Tokyo, Japan), to examine the incorporation of uridine-5-3H into RNA and thymidine-2-14C into DNA.

RESULTS

Toxicity of EB. The lethal dose of EB was examined in CBA mice. All 10 mice survived a single i.p. injection of 10 mg/kg, 2 died (lethal dose to 20% of mice) at 20 mg/kg, and 5 died (lethal dose to 50% of mice) at 40 mg/kg. With a dose of 80 mg/kg, all the animals died within 5 days.

In the toxicity test by consecutive administration for 5 days, i.p. injection of 20 mg/kg killed all the animals within 10 days, but 90% of the animals were alive at a dose of 10 mg/kg. The optimal dose, determined from these results as an i.p. injection of 8 mg/kg, was used for subsequent experiments.

Effect of EB on Various Experimental Tumors. Mice bearing L1210 were given EB, either 4 or 8 mg/kg i.p., for 5 days starting 24 hr after inoculation with 1 X 10^6 tumor cells, and essentially no prolongation of life was observed.

Mice bearing 6C3HED OG were treated with EB, 2, 4, or 8 mg/kg i.p., for 5 days starting 24 hr after tumor implantation of 1 X 10^6 cells and over 200% ILS was observed (Chart 2). However, no percentage of ILS was seen with 6C3HED RG by i.p. administration of EB, 4 or 8 mg/kg (Chart 3).

The minimum effective dose, using mice inoculated with 1 X 10^6 cells of 6C3HED OG, showed that administration of EB, either 1 or 0.5 mg/kg, for 5 consecutive days effected over 100% ILS, but there was no ILS with the administration of 0.25 mg/kg.

The effect of EB, 8 mg/kg, given i.p. on various experimental tumors is shown in Chart 4. ILS was over 200% on 6C3HED OG, 83% on L5178Y, and 51% on Sarcoma 180, whereas ILS was 20% on L1210, 3% on 6C3HED RG, 15% on EL4, 11% on RADA1, and 0% on Walker carcinosarcoma 256.

In the case of Friend virus leukemia, 70-day survival was observed in 8 of 29 animals (28%) in the control group. In the group given EB, 8 mg/kg, for 5 consecutive days, 14 of 30 animals (47%) survived, and 18 of 25 animals (72%) were alive when the same dose was administered for 10 consecutive days (Chart 5).

Antitumor Effect of Substances Related to EB. The antitumor effect of related substances was examined by using 6C3HED OG as the tumor system for screening. After i.p. transplantation of 1 X 10^6 cells of 6C3HED OG in CBA mice, 8-mg/kg doses of each of the substances listed in Chart 1 were given i.p. for 5 consecutive days. PD-MY-001 and PD-MY-003, among the 14 related substances, effected over 200% ILS (Chart 6).

The in vivo effect of PD-MY-001 and PD-MY-003 against various experimental tumors is indicated in Chart 7. The effect of PD-MY-001 on Walker carcinosarcoma 256 and Sarcoma 180 was 60 and 16% ILS, respectively, and that of PD-MY-003
Chart 2. Effect of EB on life-span of mice with 6C3HED OG lymphosarcoma. Into groups of CBA mice (10 in each group) were transplanted $1 \times 10^6$ cells of 6C3HED OG. From the next day, EB, 2, 4, or 8 mg/kg, was injected i.p. for 5 consecutive days. % along abscissa, ILS.

Chart 3. Effect of EB on life-span of mice with 6C3HED RG lymphosarcoma. Into groups of CBA mice (10 in each group) were transplanted $1 \times 10^6$ cells of 6C3HED RG. From the next day, EB, 4 or 8 mg/kg, was injected i.p. for 5 consecutive days. % along abscissa, ILS.

Chart 4. Antitumor spectrum of EB in mouse tumors with i.p. administration of EB, 8 mg/kg. %, ILS. α, 1 million ascites tumor cells were inoculated i.p. into each mouse; α′, 100,000 ascites tumor cells were inoculated i.p. into each mouse; β, 1 cu mm of solid tumor tissue was inoculated s.c. into each mouse.

was 45 and 72% ILS. For other tumors, PD-MY-003 showed no prolonging effect, ILS being 0% for L1210, 0% for L5178Y, 10% for EL4, 0% for RADA1, and 18% for Ehrlich ascites tumor. As was seen in PD-MY-001, a slight ILS effect was found only with EL4.

In Vitro Effect of EB on Various Experimental Leukemias. Observed inhibition of RNA and DNA synthesis in L1210 cells was slight with $1 \times 10^{-5}$ M EB but strong with $1 \times 10^{-4}$ M.

In 6C3HED OG cells, 50% inhibition of RNA synthesis was observed with $0.5 \times 10^{-5}$ M EB, and over 70% inhibition of RNA synthesis was observed with $1 \times 10^{-5}$ M EB. With 6C3HED RG, 50% inhibition of RNA synthesis was observed with $1 \times 10^{-5}$ M EB.

With other tumors, L5178Y and EL4, strong inhibition of both RNA and DNA synthesis was seen with $0.5 \times 10^{-4}$ M EB, but there was no marked difference between these 2 tumor strains (Table 1).
DISCUSSION

In 1971, Hirschman (5) reported the effect of various chemicals on DNA polymerase activity in murine leukemia virus. He tested 4-Af-demethyl-rifampicin, 4-A^-benzyldemethyl-rifampicin, daunomycin hydrochloride, chromomycin A3, and EB (M.W. 394) and reported that, although rifampicin derivatives acted as inhibitors of DNA polymerase in Moloney murine sarcoma virus, EB had a stronger inhibitory effect.

Since EB was found to act as a DNA polymerase inhibitor, the antitumor effects of EB and its related substances were comparatively examined. In in vitro sensitivity (6), there was no difference in the tumor-specific antitumor effect among the various tumors used; however, by in vivo examination, a life-prolonging effect was evidenced by 83 to 200% or more ILS with i.p. administration of EB, 8 mg/kg, for 5 consecutive days from the day after transplantation of 1 X 10⁶ tumor cells of 6C3HED-OG and L5178Y. An antitumor effect was not observed with 6C3HED-RG, EL4, and RADA₁. The cross-resistance between EB and the L-asparaginase-sensitive line of the 6C3HED-OG and the resistant line of RG tumor may be accounted for by the difference in the cell membranes of these 2 cell lines. Such tumor selectivity in the antitumor effect of EB in vivo could not be concluded from its test results in vitro and suggests that the mechanism of action of EB is not due merely to the difference in cell membrane.

Synthesized substances related to EB, PD-MY-001 and PD-MY-003, showed marked life prolongation against 6C3HED-OG, and slight prolongation against Walker carcino-sarcoma 256 and Sarcoma 180. This fact shows that it has become possible to develop related substances effective against other kinds of cancer through the screening of these substances and gives hope for development of an inhibitor specific to RNA-dependent DNA polymerase from among these related substances.

Sakurai (7) reported that acetylation of one of the amino groups at positions 2 and 7 of the phenanthridine ring in EB did not affect its antitumor activity but that a diacetylated derivative was ineffective; this pointed to the necessity of the presence of an amino group which takes a cationic form in a physiological environment, as do the acridines. However, Sakurai presumed that the presence of the quaternary amino

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<td>6C3HED-OG</td>
<td></td>
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<td>&gt;300</td>
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<tr>
<td>L1210</td>
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<td>L5178Y</td>
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<td>90%</td>
<td>140%</td>
<td>110%</td>
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<td>94%</td>
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<td>145%</td>
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Chart 7. Antitumor spectrum of EB-related compounds in mouse tumors. %, ILS. a, 1 million ascites tumor cells were inoculated i.p. into each mouse; a', 100,000 ascites tumor cells were inoculated i.p. into each mouse; b, 1 cu mm of solid tumor tissue was inoculated s.c. into each mouse.

Table 1
Effect of EB on RNA and DNA synthesis in murine leukemia cells in vitro

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<th>L1210</th>
<th>6C3HED-OG</th>
<th>6C3HED-RG</th>
<th>L5178Y</th>
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<td>NT⁹</td>
<td>NT⁹</td>
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<td>NT</td>
<td>NT</td>
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<td>5.8</td>
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<td>Final concentration of EB (M)</td>
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<tr>
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<td>NT</td>
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⁹ NT, not tested.
group at position 10 had no bearing on the physiological activity of EB because 2,7-diamino-10-ethyl-9-phenyldihydrophenanthridine showed the same antitumor activity as did EB. It is known that these plane molecules of N-heterocyclic compounds with amino groups often show antibacterial or antiprotozoal activity, and it may be presumed that antitumor activity would appear in these compounds if a specific structural condition were satisfied.

The plane area of the phenanthridine ring was approximately the same as that of the acridine ring (both 38 sq Å), but there was a difference in the shape of the plane, which may account for the presence of antitumor activity in the former compound. The presence of a phenyl group in position 9 of the phenanthridine ring in EB should also be taken into consideration.

Synthesis of 2,7-diaminophenanthridium of the various derivatives of phenanthridine is of interest, especially the biological activity of variously substituted derivatives of the phenyl group at position 9.

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