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Preclinical report

Antitumor activity of 2-amino-4,4α-dihydro-4α, 7-dimethyl-3H-phenoxazine-3-one against Meth A tumor transplanted into BALB/c mice

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We examined the *in vivo* effect of 2-amino-4,4α-dihydro-4α,7-dimethyl-3H-phenoxazine-3-one (Phx) on Meth A carcinoma cells transplanted into BALB/c mice, in terms of both antitumor activity and side effects. Phx, which was synthesized by the reaction of 2-amino-5-methylphenol with bovine hemolysates, was administered i.p. at doses of 1 and 5 mg/kg to BALB/c mice transplanted with Meth A tumor cells. Phx exerted a strong antitumor activity to Meth A tumor growing in the mice as 5-fluorouracil (5-FU) did. The antitumor activity of Phx at the dose of 5 mg/kg was comparable to that of 5-FU at the dose of 7.8 mg/kg. In contrast, unlike 5-FU, Phx did not cause leukopenia while showing a strong antitumor activity. The compound also produced little changes in body weight and no wasting of mice developed. These results show that Phx has strong anti-tumor activity, but exerts lower side effects and suggest that Phx is available for therapeutic purposes in the future. [© 2000 Lippincott Williams & Wilkins.]

Key words: 2-Amino-4,4α-dihydro-4,4α,7-dimethyl-3H-phenoxazine-3-one, *in vivo* anti-tumor activity, leukopenia, low side effects, phenoxazine derivative.

Introduction

It is well known that actinomycins produced by actinomyces species show a strong antitumor activity by intercalating DNA. There are many reports[1–6] examining the actinomycin analog modified phenoxazine moiety or the pentapeptide lactone rings of them to enhance the biological activity.

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In 1988, Palmer *et al.*[3] examined a number of different compounds having linear tricyclic carboxamides including phenoxazine and concluded that only the compounds containing coplanar chromophores such as phenoxazine intercalated DNA.

Motohashi *et al.*[7] in 1991 reviewed the phenoxazine and benzophenoxazine dye family, and emphasized that none of the synthetic materials prepared and tested to that date had the potency of the outstanding antitumor natural products bearing the ring system of the actinomycins. Prior to this review, Motohashi[1] examined the antitumor activity of many kinds of phenoxazine derivatives synthesized chemically, but he observed little antitumor activity in the derivatives, probably due to their poor solubility in water.

We[8,9] reported that 2-amino-4,4α-dihydro-4α,7-dimethyl-3H-phenoxazine-3-one (Phx) (see Figure 6) is easily synthesized by the reaction of 2-amino-5-methylphenol with human hemoglobin and/or bovine hemoglobin and is relatively soluble in water. Then, we[10] examined the *in vitro* effect of Phx on KB, a human epidermoid carcinoma cell line, and found that Phx inhibits cell proliferation in a dose-dependent manner, probably by inhibiting the DNA synthesis of the cells.

In the present paper, we describe the *in vivo* effect of Phx on Meth A carcinoma cells transplanted into BALB/c mice, in terms of both antitumor activity and side effects.

Materials and methods

Compounds

Phx was synthesized and purified as described previously.[10] 5-Fluorouracil (5-FU) was obtained from

Nakalai Tesk (Kyoto, Japan). The compounds were given i.p. to mice in a volume of 0.1 ml/10 g body weight. In Experiment 1, the antitumor effect of Phx was examined at doses of 1 and 5 mg/kg. The compound was dissolved in a small amount of ethanol, then suspended into 0.15 M saline (the final concentration of ethanol was less than l%). In Experiment 2, the side effects of Phx were examined at higher doses of 10 and 20 mg/kg. Since a high concentration of Phx is insoluble in ethanol, 8 mg of Phx was dissolved with 1 ml of 0.3 M HCl and then the solution was neutralized with 1 ml of 0.3 M NaOH to give a fine suspension of the compound. The resulting suspension was diluted appropriately with phosphate-buffered saline (pH 7.4) to be given to mice.

5-FU was dissolved in the corresponding vehicles. The control mice were given the vehicles.

Animals

Female BALB/c mice were obtained at 7 weeks of age from Japan SLC (Hamamatsu, Japan). The mice were maintained in the Laboratory for Animal Experiments, Gifu Pharmaceutical University, with free access to Charles River solid rodent chow (Oriental Yeast, Tokyo, Japan) and water under filtered laminar airflow conditions at $21 \pm 1^{\circ}C$, $60 \pm 5\%$ humidity and 12 h light a day. They were acclimatized for 7 days before the experiments.

Cancer cell line and transplantation

Meth A tumor cells derived from BALB/c mice were maintained as an ascites form by weekly passage in the peritoneal cavity of the syngeneic hosts. The ascitic tumor cells were washed well with Hanks' balanced salt solution by centrifugation prior to the transplantation. To examine the antitumor effect of the test compounds, 10^6 cells of the Meth A tumor were inoculated s.c. into the flank of BALB/c mice in a volume of 0.1 ml. The size of tumor growing in the subcutis was measured with Vernier calipers in terms of two diameters at right angles and expressed as volume (mm^3) calculated as follows: $^{11}4/3 \times \pi \times (\text{long diameter}/2) \times (\text{short diameter}/2)^2$.

Blood leukocyte count

Blood of mice was diluted 20 times with Türk's solution (consisting of 0.1% gentiana violet and 1% acetic acid), followed by counting leukocytes under light microscopy using a Bürker–Türk hemocytometer.

Statistics

The results were expressed as mean values $\pm$ SEM. The data for tumor size were ranked and subjected to the Kruskal–Wallis test, and then non-parametric Duncan's multiple range test (ranked multiple range test) to analyze the significance of differences between the control and the compound-treated groups. For the other data including body weight and blood leukocyte count, the parametric Duncan's multiple range test after one-way analysis of variance (ANOVA) was used to assess the statistical significance of differences between the control and the compound-treated groups. A value of $p \leqslant 0.05$ was considered to indicate a significant difference in all statistical analyses.

Results

Experiment 1: antitumor effect of Phx and 5-FU in mice transplanted with Meth A tumor

Phx at doses of 1 and 5 mg/kg or 5-FU at doses of 7.8 and 15.6 mg/kg was daily given i.p. for 14 consecutive days starting with the day of Meth A tumor transplantation (Figures 1–3).

Figure 1 shows the effects of the compounds on tumor growth. Phx suppressed the growth. Phx at 5 mg/kg reduced the tumor size to half that in the

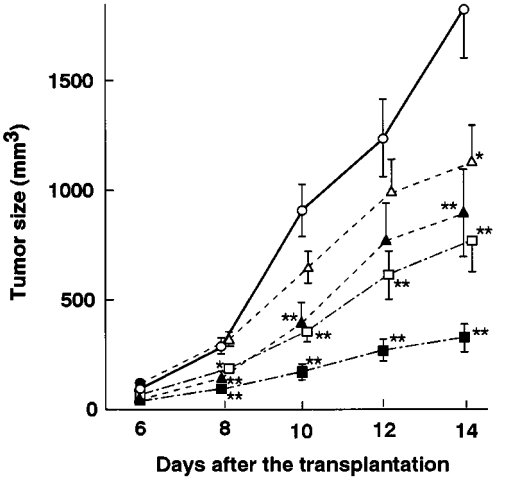

Figure 1. Effect of Phx and 5-FU on Meth A tumor growth in mice. BALB/c female mice, 8 weeks old, were transplanted s.c. with 10^6 Meth A cells on day 0 and then daily given the compounds i.p. for 14 consecutive days starting with the day of transplantation. ○, Control; △, Phx 1 mg/kg; ▲, Phx 5 mg/kg; □, 5-FU 7.8 mg/kg; ■, 5-FU 15.6 mg/kg. Each group included 10 mice except for the group dosed with Phx 5 mg/kg (nine mice). Data represent the mean $\pm$ SEM. $^*p \leqslant 0.05$; $^{**}p \leqslant 0.01$ versus control.

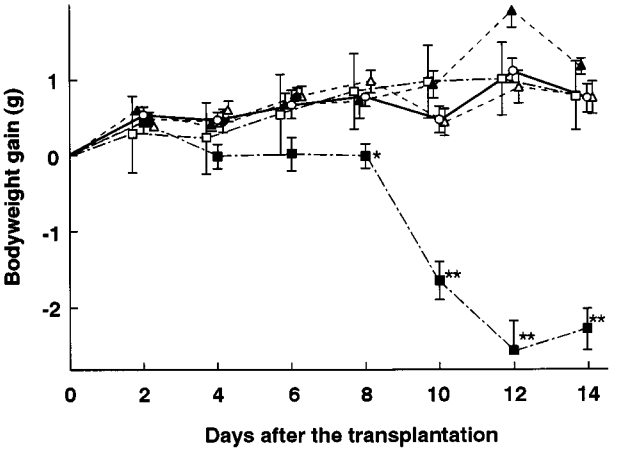

Figure 2. Effect of Phx and 5-FU on body weight of mice transplanted with Meth A cells. See legend to Figure 1 for the experimental protocol. The initial body weight of the mice was 19.3±0.14 on day 0. ○, Control; △, Phx 1 mg/kg; ▲, Phx 5 mg/kg; □, 5-FU 7.8 mg/kg; ■, 5-FU 15.6 mg/kg. Data represent the mean±SEM. *$p \leqslant 0.05$; **$p \leqslant 0.01$ versus control.

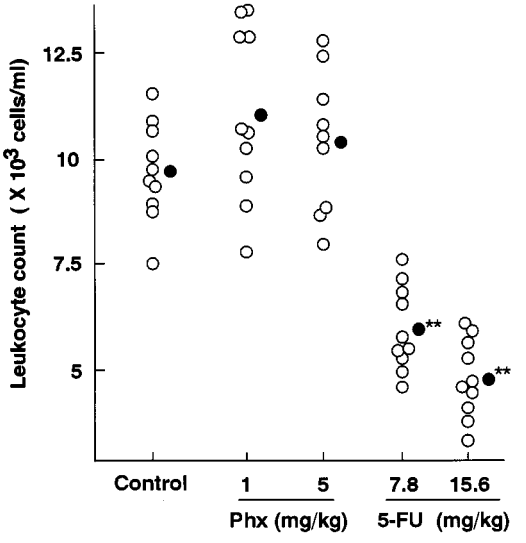

Figure 3. Effect of Phx and 5-FU on leukocyte count in blood of mice transplanted with Meth A cells. See legend to Figure 1 for the experimental protocol. Blood leukocyte counts were measured 14 days after the transplantation. Open circles represent individual mice and closed circles represent the mean of them. *$p \leqslant 0.05$; **$p \leqslant 0.01$ versus control.

control through the experimental period. 5-FU at 7.8 and 15.6 mg/kg reduced the tumor size by more than 75 and 90%, respectively, of that in the control group.

Body weight change was monitored through the experiment (Figure 2) and blood leukocyte count was measured on day 14 after the tumor transplantation (Figure 3). No significant difference was observed in body weight among the control and groups administered Phx at any doses and 5-FU at 7.8 mg/kg throughout the experiment. However, extensive reduction of body weight was seen in the group treated with 15.6 mg/kg of 5-FU for more than 8 days. No mouse died in any group during the experiment.

The blood leukocyte count on day 14 was decreased significantly by treatment with 7.8 and 15.6 mg/kg of 5-FU in comparison with the control. However, no reduction was noticed in any group given Phx.

Experiment 2: effect of Phx and 5-FU on blood leukocyte count in mice not transplanted with tumor

Since Phx has a weak or no effect on blood leukocyte count as shown in Experiment 1, we examined further the effect of Phx at higher doses on the blood leukocyte count in mice without inoculation of the tumor. Mice were i.p. given Phx at doses of 10 and 20 mg/kg or 5-FU at doses of 7.8 and 15.6 mg/kg for 14 days. The number of blood leukocytes in blood was assayed on the day after the final treatment with the compounds. Body weight was also measured and the general condition of mice monitored throughout the experiment.

The leukocyte count was confirmed not to decrease by the treatment with Phx at any dose, but to decrease drastically in mice administered 15.6 mg/kg of 5-FU, and five mice out of 10 died by day 14 (Figure 4).

There was no significant difference in body weight between the control and Phx-treated groups (Figure 5). Moreover, other side effects such as piloerection did not develop in mice treated with Phx at any dose throughout the observation period.

By treatment with 7.8 mg/kg of 5-FU, the body weight decreased during days 4 and 8, and then recovered to the level of the control. Mice treated with 5-FU at the dose of 15.6 mg/kg showed a severe reduction in body weight.

Discussion

Phx showed anti-tumor activity even at the lower dose of 1 mg/kg against Meth A tumor cells transplanted into mice. When given 5 mg/kg of Phx, the tumor reduced to almost half the size of the control—an effect comparable to that of 7.8 mg/kg of 5-FU (Figure 1).

Although 5-FU showed strong anticancer effects on Meth A tumor growing in mice, it also caused extensive piloerection, wasting of mice, loss of body

weight (Figures 2 and 5) and a decrease of leukocyte count in the blood (Figures 3 and 4). On the other hand, Phx did not show such side effects even at the dose of 20 mg/kg, which was 4 times the dose needed to exert a strong antitumor activity.

Actinomycin D is often used for children's malignant tumors, especially for Wilms tumor (kidney sarcoma). Although we did not compare the side effects of Phx with those of actinomycin D in relation to their antitumor activity, it is well known that actinomycin D has intensive side effects such as hematopoietic and immunosuppressive toxicities.

Phx, like actinomycin D, is a phenoxazine derivative, but the mechanism of growth inhibition of tumor cells may differ between the two compounds, because Phx does not intercalate DNA while actinomycin D does.[10] In the present study, we showed that Phx did not cause obvious side effects including wasting of mice and leukopenia, while showing a strong anti-

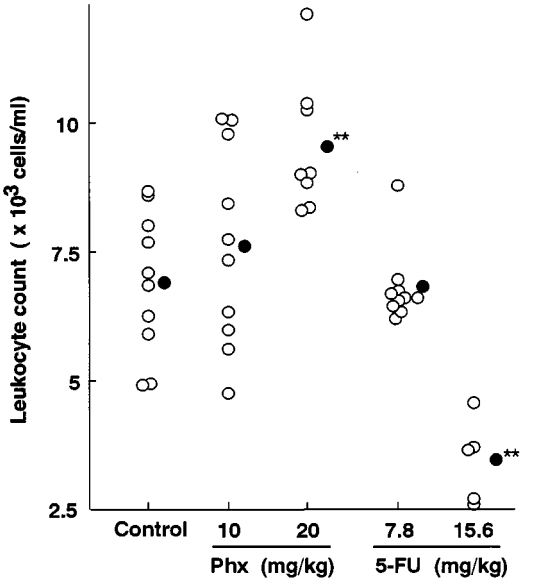

Figure 4. Effect of Phx and 5-FU on leukocyte count in blood of mice. BALB/c female mice, 8 weeks old, were daily given Phx at doses of 10 and 20 mg/kg or 5-FU at doses of 7.8 and 15.6 mg/kg i.p. for 14 consecutive days. The leukocyte counts in blood were measured the day after the final treatment with the compounds. Each group consisted of 10 mice except for eight in Phx 20 mg/kg and nine in 5-FU 7.8 mg/kg groups at the beginning. Five mice out of 10 died by the day of the final treatment with 15.6 mg/kg of 5-FU. Open circles represent individual mice and closed circles represent the mean of them. $^{**}p \leqslant 0.01$ versus control.

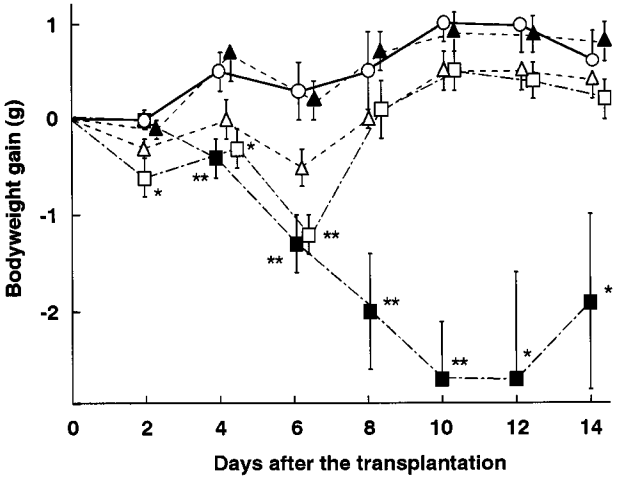

Figure 5. Effect of Phx and 5-FU on body weight of mice. See legend to Figure 4 for the experimental protocol. The initial body weight of the mice was 19.2 ± 0.13 on day 0. ○, Control; ▲, Phx 10 mg/kg; △, Phx 20 mg/kg; □, 5-FU 7.8 mg/kg; ■, 5-FU 15.6 mg/kg. Data represent the mean ± SEM. $^{*}p \leqslant 0.05$; $^{**}p \leqslant 0.01$ versus control.

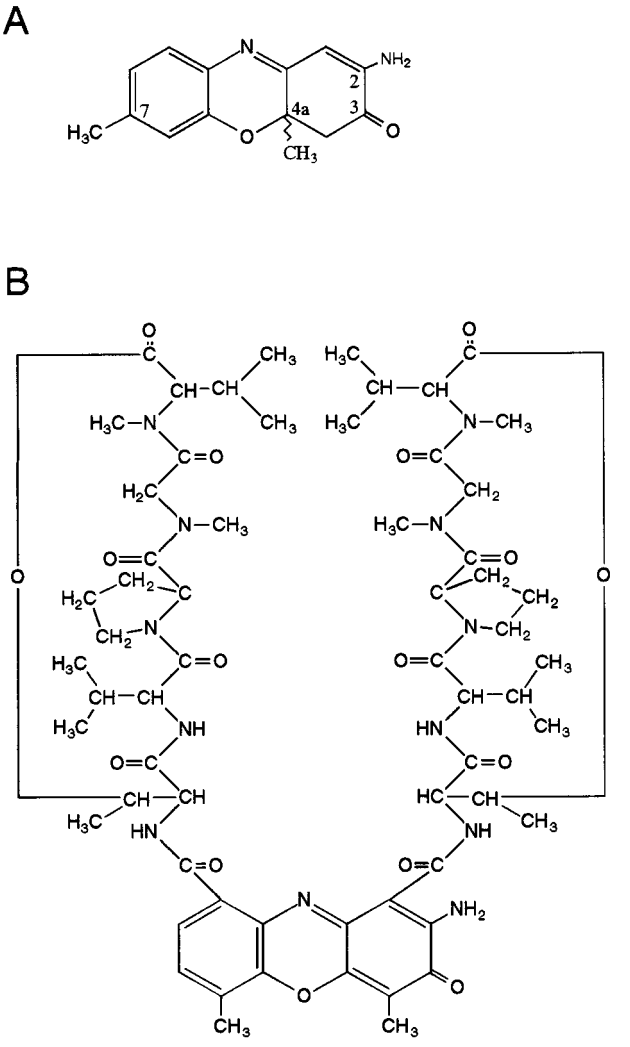

Figure 6. Chemical structure of Phx (A) and actinomycin D (B).

tumor activity. Thus the low side effects of Phx may be related to a mode of action different from actinomycin D, presumably due to the pentapeptide lactone ring including sarcosine, methylglycine, proline and valine, which actinomycin D, but not Phx, has, is necessary for the stable intercalation to DNA (Figure 6).[4–6]

Phx can be expected to be available for therapeutic purposes in the future, because Phx showed a strong antitumor activity without severe side effects.

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