ANTITUMOR EFFECT OF FORPHENICINOL, A LOW MOLECULAR WEIGHT IMMUNOMODIFIER, ON MURINE TRANSPLANTABLE TUMORS AND MICROBIAL INFECTIONS

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(Received for publication May 19, 1982)

The antitumor activities of forphenicinol against murine transplantable tumors were examined. Ehrlich carcinoma was suppressed by treatment with 0.08–0.31 mg/kg/day of forphenicinol given for 10 days starting 5 days after tumor inoculation. IMC carcinoma was also suppressed by treatment with 0.5–5 mg/kg/day given for 5 days starting 8 days after the inoculation. The antitumor activity was dependent on the number of tumor cells inoculated, schedule of administration and dose. However, even in case of fast growing tumors such as L1210 and inoculation with a large number of tumor cells, forphenicinol markedly enhanced the antitumor effect of 6-mercaptopurine, aclacinomycin and cyclophosphamide. Forphenicinol showed a protective effect on Pseudomonas infection in mice.

In a previous paper1), we reported the effect of forphenicinol on immune responses in mice and reported that forphenicinol augments delayed-type hypersensitivity (DTH), macrophage phagocytosis and the production of CFU-C in bone marrow cell cultures.

In this paper we report effects of forphenicinol on murine tumors and Pseudomonas infection.

Materials and Methods

Animals
CDF1 mice (Balb/c × DBA/2, female, 8–10 weeks old), ICR mice (female 4–8 weeks old) and ddY mice (female, 6 weeks old) were obtained from Shizuoka Laboratory Animal Agriculture Cooperative Association, Shizuoka, Japan.

Forphenicinol and Other Chemicals
Forphenicinol was chemically synthesized and dissolved in saline. Antitumor substances, aclacinomycin (Sanraku Ocean Co. Ltd., Tokyo)2,3, 6-mercaptopurine (6-MP, Sigma Chemical Co., St. Louis) and cyclophosphamide ("Endoxan" Shionogi Co. Ltd., Osaka) were dissolved in saline.

Tumors and Evaluation of Antitumor Activity
Ehrlich carcinoma and sarcoma 180 (S 180) which had been maintained by weekly intraperitoneal injection of tumor cells in ascites to ddY or ICR mice respectively, were employed as allogeneic tumors. For experiments, 2 × 10⁶ Ehrlich carcinoma cells or S 180 cells were inoculated into the groin of mice subcutaneously. In case of Ehrlich carcinoma, 15 days after tumor inoculation and in case of S 180, 30 days after the tumor inoculation, tumors were extirpated and weighed.

IMC carcinoma⁴ and murine leukemia L 1210 were used as syngeneic tumors. IMC carcinoma was maintained by weekly intraperitoneal injection of tumor cells to CDF1 mice. In experiments, 1 or 5 × 10⁶ IMC carcinoma cells were inoculated to CDF1 mouse subcutaneously and 30 days after the tumor

* Banyu Pharmaceutical Co. Ltd., 2–9–3 Shimomeguro, Meguro-ku, Tokyo 153, Japan
inoculation, tumors were extirpated and weighed. Antitumor activity was assessed by the percentage of inhibition of tumor weight caused by the treatment. For the L 1210 assay, CDF1 mice were inoculated with $5 \times 10^6$ or $1 \times 10^6$ cells intraperitoneally and the antitumor effect was evaluated in terms of T/C %.

**Pseudomonas Infection**

*Pseudomonas aeruginosa* No. 12 strain which was clinically isolated by Dr. Y. Homma, Institute of Medical Science, Tokyo University, was employed. The strain was inoculated into heart infusion broth and incubated for 18 hours at 27°C. After the incubation, the cultured broth was diluted with saline, and $3.8 \times 10^7$ cells in 0.2 ml were inoculated intraperitoneally. Forphenicinol was given orally once at the time of the infection, and 1 week thereafter, the activity was assessed by counting the number of surviving mice in the treated group compared to the non-treated group. Ten mice were used in each group.

**Results**

**Toxicity of Forphenicinol**

Acute toxicity was examined in various animals. Table 1 shows that forphenicinol has low toxicity in all routes of administration and all kinds of animals tested. Forphenicinol did not show cytotoxicity at concentration of 100 μg/ml against L 1210 cells in culture.

<table>
<thead>
<tr>
<th>Species</th>
<th>Sex</th>
<th>Route</th>
<th>No. of animals</th>
<th>Acute toxicity (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>LD_{50}</td>
</tr>
<tr>
<td>Mouse (ddY)</td>
<td>Male</td>
<td>i.v.</td>
<td>24</td>
<td>5,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>i.p.</td>
<td>40</td>
<td>3,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p.o.</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Rat (Wister)</td>
<td>Male</td>
<td>i.v.</td>
<td>20</td>
<td>5,500</td>
</tr>
<tr>
<td></td>
<td></td>
<td>i.p.</td>
<td>24</td>
<td>3,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p.o.</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Rabbit</td>
<td>Male</td>
<td>i.v.</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>i.p.</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>p.o.</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Dog (Beagle)</td>
<td>Male</td>
<td>i.v.</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>p.o.</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

**Effect on Ehrlich Carcinoma and S 180**

Mice were inoculated with $2 \times 10^6$ cells of Ehrlich carcinoma subcutaneously and 5 days after the inoculation they were given forphenicinol daily for 10 days. One day after the last administration of forphenicinol, tumors were extirpated and weighed. Fig. 1 illustrates the growth of the tumor and the tumor weight. The antitumor effect was observed with an administration of 0.08 or 0.31 μg/kg/day. The larger doses (1.25–5 mg/kg/day) did not show an antitumor effect against Ehrlich carcinoma. Forphenicinol from 0.005 to 50 mg/kg/day daily for 10 days after inoculation of tumor cells showed no antitumor activity against ascitic tumors of Ehrlich carcinoma and S 180.

The combined effect of forphenicinol with a cytotoxic substance against S 180 was examined. Mice were inoculated with $2 \times 10^6$ S 180 cells subcutaneously and 10 mg/kg of 6-MP was injected daily from day 1 to 10 after the inoculation. Forphenicinol was given orally from day 11 to 15. Thirty days after
the tumor inoculation, the effect was assessed by weighing tumors. As shown in Table 2, 6-MP alone was not effective in suppressing the growth of the tumor, but 6-MP with forphenicinol from 0.005 to 5 mg/kg/day was effective. The most effective dose of forphenicinol was 0.05 to 0.5 mg/kg/day given with 10 mg/kg/day of 6-MP. Forphenicinol alone also showed a weak antitumor activity.

Effect on IMC Carcinoma

Mice were inoculated with $1 \times 10^6$ IMC carcinoma cells, and forphenicinol was given intraperitoneally or orally from 8 to 12 days after the tumor cell inoculation. Thirty days after the inoculation of tumor cells, the effect was evaluated by weighing the tumors. The result is shown in Table 3. The administration of 0.5 to 5 mg/kg/day of forphenicinol suppressed growth of the tumor by 70~80%. The effect was observed in both routes. However, when a large number of IMC carcinoma cells (larger than $2 \times 10^6$ IMC carcinoma cells) were inoculated, forphenicinol was not effective. It was also not effective against the ascites form of IMC carcinoma.

Mice were inoculated with $5 \times 10^6$ IMC carcinoma cells subcutaneously and aclacinomycin A (0.005 mg/kg/day, i.p.) was given at 1, 3, 5, 7 and 9 days after the inoculation. In one group, forphenicinol (0.05 mg/kg/day, p.o.) was given daily for 10 days from 1 day after the inoculation. As shown in Table 4, aclacinomycin showed only weak antitumor effect (25% inhibition) and this effect was augmented to 48% ($P < 0.05$) by the administration of forphenicinol. Forphenicinol alone did not show any inhibitory effect.

Effect on L 1210 with or without Cyclophosphamide

Mice were inoculated with $1 \times 10^6$ L 1210 cells intraperitoneally, and forphenicinol was given intraperitoneally, daily for 10 days from 1 day after the inoculation. The effect was assessed by the survival...
periods. As shown in Table 5, forphenicinol did not show any effect on prolongation of the survival period.

The effect of forphenicinol in combination with cyclophosphamide against L 1210 was examined. Mice were inoculated with $5 \times 10^6$ L 1210 cells intraperitoneally, and 1 day after the inoculation, 50 mg/kg of cyclophosphamide was injected intraperitoneally once. Starting two days after the inoculation, forphenicinol was given daily for 10 days. As shown in Table 6, cyclophosphamide alone prolonged the survival period (142% in T/C %) but no mice survived 30 days after the inoculation. Forphenicinol enhanced the antitumor effect of cyclophosphamide and prolonged the survival period (169 ~ 196%). Moreover, 3 of 6 mice survived 30 days after the inoculation. The optimum dose of forphenicinol was from 0.1 to 1 mg/kg/day.
Effect on *Pseudomonas* Infection

Mice were injected with $3.8 \times 10^7$ cells of *Pseudomonas aeruginosa* No. 12 intraperitoneally, and forphenicinol was given orally to mice immediately after the infection. A number of cells which caused death of about 70% of the mice was chosen to test the effect of forphenicinol. One week after the infection, the number of surviving mice was counted. As shown in Table 7, 3 out of 10 mice survived in the non-treated control and from 6 to 9 out of 10 mice survived in the forphenicinol-treated group in doses from 15.6 μg to 1 mg/mouse. Doses higher than 1 mg or lower than 15.6 μg, did not show a protective effect. Forphenicinol from 1 to 1,000 μg/ml did not show any effect in inhibiting the growth of *Pseudomonas aeruginosa* No. 12 or other bacteria.

### Table 7. Effect of forphenicinol against infection of *Pseudomonas aeruginosa* in mice.*

<table>
<thead>
<tr>
<th>Forphenicinol (μg/mouse, p.o.)</th>
<th>Survived/treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>4,000</td>
<td>4 / 10</td>
</tr>
<tr>
<td>1,000</td>
<td>6 / 10</td>
</tr>
<tr>
<td>250</td>
<td>7 / 10</td>
</tr>
<tr>
<td>62.5</td>
<td>8 / 10</td>
</tr>
<tr>
<td>15.6</td>
<td>9 / 10</td>
</tr>
<tr>
<td>3.9</td>
<td>1 / 10</td>
</tr>
<tr>
<td>0</td>
<td>3 / 10</td>
</tr>
</tbody>
</table>

* ICR mice (male, 4 weeks old) were inoculated with $3.8 \times 10^7$ cells of *P. aeruginosa* No. 12 intraperitoneally and given forphenicinol once orally. The number of survivors was determined 7 days after the inoculation.

### Discussion

In a previous paper[^1], we reported that forphenicinol augments cell-mediated immunity and stimulates macrophage phagocytosis and production of CFU-C in bone marrow cell cultures. Forphenicinol has extremely low toxicity in animals and cultured mammalian cells. In this report, we examined the effect of forphenicinol against various murine tumors. As allogeneic tumors, Ehrlich carcinoma and S 180 were employed to test its effect. The subcutaneous solid tumors of both Ehrlich carcinoma and S 180 were suppressed by forphenicinol, but the ascitic forms were not. Forphenicinol treatment started from 5 or 8 days after inoculation and continued for 5 days was effective, but when started from 1 day after the inoculation for 10 days it was not effective. Forphenicinol at doses of 0.08 ~ 0.31 mg/kg/day started 5 days after the inoculation was effective but not 1.25 mg/kg/day against Ehrlich carcinoma. As shown by these experiments, the antitumor effect of forphenicinol is dependent on the schedule of administration and the dose. The effect of forphenicinol 5 or 8 days after the tumor cell inoculation may be due to the effect on concomitant immunity[^2] to the tumor already produced. It was also effective against syngeneic tumors. Against IMC carcinoma, forphenicinol alone exhibited inhibition and the effect was dependent on the administration schedule. If the treatment was started 1 day after inoculation, forphenicinol did not suppress the growth of this tumor. We have observed that the antitumor effect of bestatin against IMC carcinoma was also dependent on the schedule of the administration, the number of tumor cells inoculated and the dose[^3]. The antitumor activity of forphenicinol did not appear when a number of IMC carcinoma cells larger than $2 \times 10^6$ cells were inoculated. The ineffectiveness of forphenicinol when a large number of tumor cells were inoculated may be due to the fast growth of the tumors. The effect of forphenicinol suggests that the antitumor effect of forphenicinol is due to macrophage activation[^4], augmentation of concomitant immunity[^5] and probably NK cell activation[^6].

In mice inoculated with a large number of cells of a fast growing tumor such as L 1210, forphenicinol was shown to be effective in combination with a cytotoxic substance. The results shown in Tables 2, 4 and 6 indicate that the antitumor effect of 6-MP on S 180, aclacinomycin on IMC carcinoma and cyclophosphamide on L 1210 were significantly enhanced by the administration of forphenicinol. As reported in a previous paper[^7], forphenicinol stimulates CFU-C in bone marrow cell cultures. The enhancement of the antitumor effect of cytotoxic agents by forphenicinol may be due to the restoration of defense mechanisms damaged by the cytotoxic agents. There is also the possibility that the destruction or inhibition of the generation of suppressor cells by cytotoxic agents[^8] may be responsible to the anti-
tumor effect of forphenicinol.

The influence of forphenicinol on microbial infection in mice was examined and the forphenicinol exhibited a protective effect against a weak infection of *Pseudomonas aeruginosa*. This effect can be expected from its effect in enhancing macrophage phagocytosis.

Its low toxicity, its effect on the mouse immune system and its antitumor effects make forphenicinol a worthy candidate for evaluation in the treatment of cancer and infections in patients with immunodeficiencies.

Acknowledgements

This work was partly supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan and by a grant from the Ministry of Health and Welfare, Hepatitis Research Committee, Japan, and by a contract from the Division of Cancer Treatment, the National Cancer Institute, NO1-CM-57009, U.S.A.

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