ANTITUMOR EFFECT OF FORPHENICINOL, A LOW MOLECULAR WEIGHT IMMUNOMODIFIER, IN COMBINATION WITH SURGERY ON METH A FIBROSARCOMA, LEWIS LUNG CARCINOMA, AND ADENOCARCINOMA 755

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Forphenicinol, *S*-2-(3-hydroxy-4-hydroxymethylphenyl)glycine, prolonged the survival period of mice implanted with Meth A fibrosarcoma or Lewis lung carcinoma. The effect was observed in the mice when the primary tumor was removed by amputation. At autopsy, metastasized tumors were found in all dead mice but no tumor foci were detected in any of the mice still alive at the time of sacrifice. Moreover, forphenicinol suppressed the growth of subcutaneously implanted adenocarcinoma 755. When combined with surgical resection of primary tumors, forphenicinol administration both before and after resection suppressed the growth of recurrent tumors and prolonged the survival period of the mice.

Forphenicinol is effective in inhibiting the growth of various murine tumors[1,2], and this effect may be due to macrophage activation[3]. It has been shown that activated macrophages can suppress the metastasis of experimental tumors[4]. It is possible that macrophages minimize the growth of residual tumor cells after surgical removal of the primary tumors. In this paper, we report the effect of forphenicinol on metastatic and recurrent tumors.

Materials and Methods

Animals

Specific pathogen-free female BDF_1 mice (C57BL/6×DBA/2) and CDF_1 mice (BALB/c×DBA/2) were purchased from Charles River Japan Inc. (Kanagawa) and Shizuoka Laboratory Animal Center (Shizuoka). They were maintained in a barrier system, and were 6 weeks old at the start of each experiment.

Forphenicinol and Other Reagent

Forphenicinol was synthesized by Banyu Pharmaceutical Co., Ltd. according to the method described by MORISHIMA *et al.*[5]. Mitomycin C was purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo).

Tumors and Antitumor Activity Evaluation

Experiments were performed with 3 transplantable murine tumors, Meth A fibrosarcoma (Meth A), Lewis lung carcinoma (3LL), and adenocarcinoma 755 (Ca 755). 3LL and Ca 755 cells were maintained by biweekly subcutaneous passage in C57BL/6 mice (Charles River Japan Inc.) and BDF_1 mice, respectively. Single tumor cell suspensions were prepared by passing finely chopped tumor tissues successively through an 80- and a 150-mesh wire sieve. The viability of tumor cells in both cases

was approximately 60% based on trypan blue dye exclusion. Meth A cells were kept in BALB/c mice (Charles River Japan Inc.) by weekly intraperitoneal passage.

Antitumor activity of forphenicinol was evaluated by determination of survival period, incidence of cured mice, and tumor size. With combination therapy of drug and surgery, survival time after surgery was measured. All mice, including survivors, were subjected to autopsy in order to determine the number of metastasized tumor nodules in lungs under a dissecting microscope after Bouin's fixation[6].

Surgery

Mice bearing Meth A or 3LL cells which had been injected into a hindleg footpad were anesthetized with ethyl ether, and the legs with tumors of 8 to 10 mm in diameter were disinfected with 70% alcohol. Thereafter, tumor-bearing legs were amputated with scissors, and the wound was coated with an instant adhesive agent. A rubber band was used to stop bleeding. After amputation the mortality was less than 5%, and neither primary tumor recurrence nor bacterial infection was evident. In the case of Ca 755, tumor cells were inoculated subcutaneously into the flank of each mouse. When the tumors grew to approximately 14 mm in diameter, they were totally resected under anesthesia and disinfected as described above. The wound was covered with the remaining skin and treated with an adhesive agent. The mortality from surgery was less than 2%. Bacterial infection was not observed.

Results

Antitumor Effect on Meth A fibrosarcoma

CDF_1 mice were inoculated subcutaneously with 1×10^6 Meth A cells into their right flanks on day 0. They were treated by oral administration of 0.1, 1.0, or 10 mg/kg of forphenicinol once a day for 10 days from day 1 to 10. Forphenicinol administration of 1 mg/kg/day prolonged the survival period of the mice by 19%. However, the drug was ineffective against the growth of primary tumors (Table 1). In this experiment, mitomycin C, as a reference drug, inhibited the growth of primary Meth A tumors by 62% at a dose of 1 mg/kg/day, but its effect on the survival time was almost the same as that of 1 mg/kg/day of forphenicinol.

The antitumor effect of forphenicinol in combination with surgical amputation on Meth A tumors was also examined. CDF_1 mice were implanted subcutaneously with 1×10^6 Meth A cells into a footpad of their right hindlegs on day 0. Induced primary tumors were removed on day 14. Mice were treated with various doses of forphenicinol once a day for 10 consecutive days starting 1 day after the tumor implantation (Table 2, expt 1). Treatment with 0.1 or 1.0 mg/kg/day of forphenicinol increased the cure rate from 36% in the absence of treatment to 67%. In the case when forphenicinol was administered once a day for 6 days from day 8 to 13 at doses of 0.1 or 1.0 mg/kg/day, it prolonged the survival time of mice by 120 or 73%, respectively (Table 2, expt 2). Metastatic tumor nodules were found in the lungs and lymph nodes of all dead mice, but not in the survivors. This effect of forphenicinol against the metastatic Meth A was comparable to intraperitoneal injection of mitomycin C.

Antitumor Effect against Lewis Lung Carcinoma

BDF_1 mice were subcutaneously inoculated with 1×10^6 3LL cells into their footpads on day 0. The legs with tumors were removed on day 9. Mice were treated by oral administration of 0.1, 1.0, and 10 mg/kg of forphenicinol once a day for 10 days from day 7 to 16. As shown in Table 3, mice in the control group died with tumors between 12 and 24 days after surgery. After treatment with 10 mg/kg/day of forphenicinol, however, 2 out of 6 mice were still alive on day 60. These survivors were sacrificed in order to examine their lungs for metastases; no metastasized tumor nodules were observed.

Table 1. Antitumor effect of forphenicinol on Meth A.

Compound	Treatment (day 1~10)		Tumor size (day 21)	Survival days		
	mg/kg	Route	$mm^2 \pm SE$	Median	T/C (%)	Range
None			401±32	39.5	100	34~52
Forphenicinol	0.1×10	po	377±48	41.0	104	34~57
〃	1.0×10	〃	460±20	47.0	119	37~53
〃	10.0×10	〃	486±10	42.5	105	37~53
Mitomycin C	0.5×10	ip	313±30	43.5	110	34~49
〃	1.0×10	〃	153±32	46.0	116	35~71

Mice: CDF_1 female, 6 weeks old, $n=6$ (control, 12).
Tumor: 1×10^6 cells/mouse sc on day 0.

Table 2. Antitumor effect of forphenicinol in combination with amputation of Meth A.

	Treatment			Survival days No. of		
	Compound	Route	mg/kg	Median	T/C (%)	Cured mice
Expt 1						
	None			39.0	100	4/11
	Forphenicinol	po	0.01×10	40.5	104	2/6
	〃	〃	0.1 ×10	75.0	192	4/6
	〃	〃	1.0 ×10	75.0	192	4/6
	〃	〃	10.0 ×10	73.5	188	3/6
	Mitomycin C	ip	1.0 ×10	75.0	192	5/6
Expt 2						
	None			15.0	100	0/5
	Forphenicinol	po	0.01× 6	14.0	93	0/5
	〃	〃	0.1 × 6	33.0	220	1/5
	〃	〃	1.0 × 6	26.0	173	0/5
	〃	〃	10.0 × 6	17.5	117	0/5
	Mitomycin C	ip	0.1 × 6	27.5	185	0/5

Mice: CDF_1 female, 6 weeks old.
Tumor: 1×10^6 cells/mouse footpad sc on day 0.
Treatment: Expt 1, day 1 to 10; expt 2, day 8 to 13.
Amputation: Expt 1, day 14; expt 2, day 13.

The number of tumor foci in the lungs of dead mice was 120±64 (SD). There was no difference between treated- and non-treated-groups.

Antitumor Effect against Adenocarcinoma 755

BDF_1 mice were subcutaneously implanted with 3×10^5 Ca 755 cells into their flanks and divided into 2 groups on day 0. Mice of one group were treated with various doses of forphenicinol once a day for 10 days from day 4 to 13 (pre-treatment), and their tumors were then resected on day 14. Mice in the other group were subjected to the resection on day 14, and forphenicinol was given once a day for 10 consecutive days starting one day after the surgery (post-treatment). As shown in Table 4, pre-treatment with 0.1 mg/kg/day of forphenicinol inhibited the growth of primary tumors by 36% ($P<0.05$ by Student's t-test). Post-treatment also suppressed relapse and prolonged the survival time of mice, but the effect was not significant. The effect of pre-treatment on recurrent tumors was more pronounced than that of post-treatment. Forphenicinol administration at doses of 0.1 or 1.0 mg/kg/day suppressed the growth of recurrent tumors by 52 or 63%, respectively. Moreover, they increased the

Table 3. Antitumor effect of forphenicinol in combination with amputation on Lewis lung carcinoma.

Forphenicinol mg/kg, po (day 7~16)	Survival days			No. of cured mice (day 60)
	Median	T/C (%)	Range	
—	17.0	100	12~ 24	0/10
0.1×10	18.0	106	15~>50	1/6
1.0×10	19.0	112	14~>50	1/6
10.0×10	23.0*	135	18~>50	2/6

* $P<0.05$ by U-test.
Mice: BDF_1 female, 6 weeks old.
Tumor: 1×10^6 cells footpad sc on day 0.
Amputation: Day 9.

Table 4. Antitumor effect of forphenicinol on adenocarcinoma 755.

Forphenicinol po		Primary tumor size day 14		Recurrent tumor size day 21		Survival days after amputation	
mg/kg	Schedule	$mm^2\pm SD$	Inhibition (%)	$mm^2\pm SD$	Inhibition (%)	Median	Range
0	—	598±171	0	457±252	0	10.0	9~23
0.01	Day 4~13	443±187	26	366±205	20	21.0	12~38
0.1	〃	382±200*	36	221±261	52	24.0*	13~42
1.0	〃	494±138	17	170±231	63	13.0	11~19
10.0	〃	578±103	3	364± 75	20	10.5	10~21
0.01	Day 15~24	—		311±298	32	12.0	9~22
0.1	〃	—		353±273	23	13.0	10~22
1.0	〃	—		393±223	14	14.0	10~20
10.0	〃	—		305±224	33	19.0	10~21

* $P<0.05$ by Student's t-test and U-test.
Mice: BDF_1 female, 6 weeks old.
Tumor: 3×10^5 cells/mouse side flanks sc on day 0.
Amputation: Day 14.

survival period of mice by 140 and 30%.

Discussion

As described in this paper, oral administration of forphenicinol increased the survival period of mice implanted with Meth A and 3LL tumor cells when combined with surgical treatment. In the course of these experiments, the timing of the amputation was very critical; when the amputation was made at a late stage of tumor development, the effect of forphenicinol was moderate. On the other hand, when amputation was carried out at an early stage, a few mice were cured by the surgery alone, and the effect of forphenicinol treatment in addition to surgery was additive. Many mice were cured by forphenicinol administration. These results suggest that forphenicinol affects metastatic tumors at the stage between the liberation of tumor cells from the primary tumors and the proliferation of metastasized cells. Forphenicinol was also effective in suppressing the growth of residual Ca 755 cells after the surgical resection of the primary tumors.

As reported elsewhere[3], forphenicinol activates macrophages; moreover NITTA *et al.* has shown that forphenicinol augments the concomitant immunity towards tumors in mice[2]. As for the prevention of recurrence, in addition to activation of macrophages, we suppose that the concomitant immunity enhanced by forphenicinol plays a role in suppressing the secondary tumor growth, since treatment with forphenicinol before surgery was more effective than the treatment after surgery.

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