OF4949, NEW INHIBITORS OF AMINOPEPTIDASE B

V. EFFECT ON THE MURINE IMMUNE SYSTEM

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OF4949-I inhibited the growth of the solid form of IMC carcinoma and protected against pulmonary metastases of Lewis lung carcinoma. It also augmented the cytostatic activity of mouse peritoneal macrophages, the natural killer activity, and the antibody-dependent cell-mediated cytotoxicity of mouse spleen cells. This substance was not cytotoxic to cultured tumor or normal cells even at high concentrations. These results suggest that a cell-mediated immune response stimulated by this compound might account for its antitumor activity.

OF4949-I and II are inhibitors of aminopeptidase B from Ehrlich ascites carcinoma (EAC). They were isolated from the culture broth of *Penicillium rugulosum* OF4949 and identified as novel cyclic peptides containing diphenyl ether as a chromophore.[1,2] They are selective inhibitors of aminopeptidase B, leucine aminopeptidase, and enkephalin-degrading aminopeptidase.[3] These inhibitors enhance the delayed-type hypersensitivity (DTH) of mice to sheep red blood cells (SRBC), and have very low toxicity.[1] As reported by Umezawa *et al.*,[4~9] various inhibitors of aminopeptidase enhance the immune response. Amastatin increases the number of antibody-forming cells in mouse spleen; bestatin, arphamenines A and B, α-aminoacyl arginine, and diprotins A and B enhance DTH and other cell-mediated immune responses. Bestatin and arphamenines A and B have antitumor activity in animals. So we thought that I and II might enhance immune responses directed against tumor cells.

Here, we report on the antitumor activity and the immuno-potentiating activity of I.

Materials and Methods

OF4949-I and II were prepared from the fermentation broth of *Penicillium* strain No. M414.[1,10]

Animals

CDF_1 mice (BALB/C×DBA/2, females, 8~15 weeks old), BDF_1 mice (C57BL/6×DBA/2, females, 4~6 weeks old), and C57BL/6 mice (males, 8~10 weeks old) were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu, Japan. The ICR mice (females, 4~6 weeks old) were from Clea Japan, Inc., Tokyo, Japan.

Cell Lines

IMC carcinoma was kindly provided by the Institute of Microbial Chemistry, Tokyo. We maintained IMC carcinoma in CDF_1 mice by ip inoculation every 7 days. Lewis lung carcinoma (3LL), the kind gift of Dr. Shigeru Tsukagoshi of the Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, Tokyo, was maintained by biweekly sc transplantation in C57BL/6 mice.

The cultured cell lines L5178Y (mouse leukemia) and YAC-1 (mouse Molony leukemia) were kindly provided by the National Cancer Center Research Institute, Tokyo. C3H mouse embryonic kidney cells (C3H-2K), the SV40-transformed cell line of C3H-2K (SV-C3H), Fisher rat embryonic

cells (3Y1), the SV40-transformed cell line of 3Y1 (SV-3Y1), NIH mouse embryonic cells (NIH-L1), the 4NQO-transformed cell line of NIH-L1 (L1-4NQO), Swiss mouse embryonic cells (3T3), and Chinese hamster ovary cells (CHO/Pro^-) were kindly provided by Dr. KAZUKIYO ONODERA of the University of Tokyo, Department of Agricultural Chemistry.

Antitumor Effect

First, 4×10^6 cells of IMC carcinoma were implanted sc into the flank of CDF_1 mice. OF4949-I was given ip daily for 7 days on different schedules.

To test the effect of I on secondary transplanted tumors, 2×10^6 cells of IMC carcinoma were inoculated into the left hind footpad of CDF_1 mice. Three days before the inoculation of the second tumor, the first tumor was removed by amputation of the foot. Fourteen days after the inoculation of the first tumor, 10^5 of the tumor cells were transplanted by ip injection, and I was given ip daily for 7 days from 24 hours after this second inoculation.

Anti-metastatic Activity

Lewis lung carcinoma cells (2×10^5 viable cells) were prepared[11] and implanted sc in the left hind footpad of BDF_1 mice. On day 15, the foot bearing the first tumor was surgically amputated. Metastatic pulmonary nodules were counted on day 28. OF4949-I was given ip daily from day 1 to 11.

Antibody Formation

CDF_1 mice were immunized with 10^8 SRBC by iv injection, and I was given ip at the same time. Four days later, the numbers of plaque-forming cells (PFC) among mouse spleen cells were measured by the hemolytic plaque technique[12] with SRBC and guinea pig complement.

Cytostatic Assay

Tumor-free CDF_1 mice were given I ip. Four days later, peritoneal cells (PC) were harvested and cultured in RPMI 1640 medium with L5178Y leukemia cells as their target for 3 days; 18 hours before the assay, [^{3}H]thymidine ([^{3}H]TdR) was added to the culture. The cytostatic activity was assayed by the inhibition of [^{3}H]TdR incorporation. Cells adhering to plastic were prepared by the method of KUMAGAI *et al.*[13]

To examine the cytostatic activity of PC from mice bearing IMC carcinoma, such cells (7.5×10^6) were transplanted sc in CDF_1 mice, and I was injected ip into the mice on day 10. PC were taken from the mice 14 days after the tumor inoculation and assayed *in vitro*. IMC carcinoma cells in RPMI 1640 medium were cultivated together with PC for 48 hours.

The percentage of inhibition of tumor cell proliferation was calculated with the following formula.

$$\text{Inhibition}\,(\%)=100-\frac{[^3\text{H}]\text{TdR uptake when target cells were cultured with effector cells (cpm)}}{[^3\text{H}]\text{TdR uptake with cultured target cells only (cpm)}}\times100$$

Cytotoxic Assay

To examine the influence of I on natural killer (NK) activity, I was injected ip into CDF_1 or C57BL/6 mice. One to 7 days later, non-adherent mouse spleen cells were prepared and assayed for NK activity against YAC-1 as the target in a ^{51}Cr release assay.[14]

To study the effects of I on the antibody-dependent cell-mediated cytotoxicity (ADCC), various doses of I were given ip to CDF_1 mice daily for 5 days. Twenty-four hours after the last injection, the ADCC activity of whole spleen cells was assayed with chicken red blood cells (CRBC) as the target by the method of HINUMA *et al.*[15]

Assay of Colony-forming Units in Culture (CFU-C)

Bone marrow cells of CDF_1 female mice were cultured in α-MEM with colony-stimulating factor based on the method of METCALF[16] and STANLEY *et al.*[17] After 10 days of incubation, CFU-C was counted by the method of ISHIZUKA *et al.*[6] Serum induced by lipopolysaccharide (LPS) was used as the colony-stimulating factor. OF4949-I was added at various concentrations at the start of the culture.

Table 1. Effects of OF4949-I on the solid form of IMC carcinoma on different schedules.

OF4949-I (mg/kg/day)	Days −7 to −1		Days 0 to 6		Days 7 to 13	
	Tumor weight (mean±SD)	IR (%)	Tumor weight (mean±SD)	IR (%)	Tumor weight (mean±SD)	IR (%)
0 (Control)	4.0±2.4	0	5.7±2.3	0	4.4±2.5	0
0.05	4.1±1.9	−3	4.0±1.2	31	2.8±2.5	35
0.5	3.8±1.7	5	3.6±1.8	38	2.0±1.7*	55
5	3.9±0.8	−2	2.1±1.0*	64	1.5±1.0*	68
50	ND		3.1±2.0	46	4.0±1.2	8

Mice were killed and tumors were weighed on day 28.
ND: Not done.
Student's t-test compared with control (control, $n=7$; experimental, $n=7$).
* $P<0.05$.

Results

Effects on Murine Transplantable Tumors

The antitumor effects of I against the solid form of IMC carcinoma were examined. OF4949-I inhibited the growth of the tumors by ip injection from days 0 to 6 or from days 7 to 13 at doses of 0.5 or 5 mg/kg/day, but not by treatment from days −7 to −1 (Table 1). To test the antitumor activity against the ascites form of IMC carcinoma, 1×10^5 of the tumor cells were transplanted by ip injection. OF4949-I did not affect life span at dosages from 0.05 to 5.0 mg/kg/day for 7 days. The effect of I on secondary transplanted IMC carcinoma was next tested on the same treatment schedule as above. Injection of 0.5 or 5.0 mg/kg/day of I for 7 days prolonged survival time by 30% to 60%.

The anti-metastatic effect of I was examined with Lewis lung carcinoma inoculated sc into the footpad. The number of pulmonary nodules decreased at doses of from 1.0 to 5.0 mg/kg/day for 11 days (Table 2).

Table 2. Effects of OF4949-I on lung colony formation by footpad inoculation of 3LL cells.

OF4949-I (mg/kg/day)	Mean No. of lung nodules/mouse (mean±SD)	IR (%)
0 (Control)	11.4±11.6	0
0.2	12.8±18.8	−12
1.0	5.5±4.4	52
5.0	1.7±2.3*	85

Student's t-test compared with control (control, $n=15$; experimental, $n=8$).
* $P<0.05$.

To elucidate the antitumor mechanism of I, we examined cell-growth inhibition by I and II using cultured neoplastic or normal cell lines (L5178Y, YAC-1, SV-C3H, SV-3Y1, L1-4NQO, CHO/Pro⁻, 3T3, C3H-2K, 3Y1, and NIH-L1). In contrast to the results *in vivo*, I did not inhibit growth of any of the cultured cells tested at the concentration of 100 μg/ml. These results indicated that the antitumor effects of I were not due to direct cytotoxicity.

Effects on Formation of Antibodies to SRBC

The effects of I and II on antibody formation to SRBC were tested by the hemolytic plaque technique. Administration of I increased the number of antibody-forming cells by about 30% at doses of from 0.05 to 5.0 mg/kg, but the increases were not statistically significant.

Effect on Macrophage, NK and ADCC Activity

We examined the effects of I on the cytostatic activity of peritoneal macrophages. The intraperitoneal administration of I augmented the cytostatic activity of PC in doses of 0.05 to 5.0 mg/kg. The

cell fraction of PC from I-treated mice that adhered to plastic was very active, but [^{3}H]TdR incorporation into tumor cells was almost not inhibited at all by the I-treated non-adherent cell fraction. Furthermore, the activity of whole PC treated with I was not eliminated by treatment with anti-Thy 1.2 serum and complement. These results suggested that I augmented the cytostatic activity of PC through the stimulation of macrophages.

The effect on the cytostatic activity of PC from mice bearing IMC carcinoma was next examined.

Table 3. Effects of OF4949-I on cytostatic activity of mouse peritoneal cells in IMC carcinoma-bearing mouse.

Compound	Dose (mg/kg)	% Inhibition (mean±SD)		
		E/T 50	E/T 25	E/T 12.5
OF4949-I	0 (Control)	51±2	13±1	0±0
	0.5	80±6*	55±5*	36±2*
	5.0	72±5*	55±4*	42±3*

[^{3}H]TdR uptake with cultured target cells only=46,986±3,981 cpm.
Student's t-test compared with control (control, n=6; experimental, n=6).
E/T: Effector/target ratio.
* P<0.01.

Table 4. Effects of OF4949-I on NK activity of mouse spleen cells.

Compound	Dose (mg/kg)	% Specific lysis (mean±SD)			
		E/T 100	E/T 50	E/T 25	E/T 12.5
OF4949-I[a]	0 (Control)	10.6±1.6	6.8±1.0	2.6±0.7	1.7±0.6
	0.05	6.3±0.9	4.5±0.8	2.4±0.9	1.2±0.6
	0.5	21.0±2.7**	14.2±1.6**	7.6±2.2**	3.7±0.8**
	5.0	28.1±3.4**	20.0±1.8**	11.7±4.4**	7.9±1.8**
OF4949-I[b]	0 (Control)	11.6±0.2	9.3±1.0	6.1±1.3	3.7±0.9
	0.031	12.3±0.6	9.0±0.6	6.0±0.5	3.8±0.1
	0.125	14.2±1.7*	10.6±0.6	6.7±0.8	3.8±0.4
	0.05	15.6±1.2*	10.3±0.5*	7.5±0.5	4.3±0.4
	0.2	18.9±3.0**	15.1±2.4**	11.3±0.9**	5.9±0.5
	1.0	17.1±1.0*	12.8±1.2*	8.5±0.6	4.9±0.8

[a] C57BL/6. Maximum release=2,651±49 cpm. Spontaneous release=154±5 cpm.
[b] CDF_1. Maximum release=3,255±83 cpm. Spontaneous release=231±20 cpm.
Student's t-test compared with control (control, n=6; experimental, n=3).
E/T: Effector/target ratio.
* P<0.05, ** P<0.01.

Table 5. Effects of OF4949-I on ADCC activity of mouse spleen cells.

Compound	Dose (mg/kg/day)	% Specific lysis (mean±SD)		
		E/T 12.5	E/T 6.25	E/T 3.13
OF4949-I[a]	0 (Control)	38.0±2.0	15.8±0.9	10.3±2.2
	0.05	49.5±2.0*	28.6±2.0*	21.6±2.8*
	0.5	46.5±3.5*	29.2±2.7*	22.8±2.4*
	5.0	62.0±4.0*	43.2±2.8*	27.4±2.1*

[a] Maximum release=6,009±196 cpm. Spontaneous release=290±9 cpm.
Student's t-test compared with control (control, n=6; experimental, n=3).
E/T: Effector/target ratio.
* P<0.01.

Administration of I increased the cytostatic activity of the PC even from tumor-bearing mice (Table 3).

To examine the effects on NK activity, the cytotoxic activity of mouse spleen cells against YAC-1 cells was measured daily for 7 days after a single injection of I. The NK activity peaked at 3 days after ip injection of I. Administration of from 0.5 to 5.0 mg/kg of I caused a 1.5- to 2.5-fold increase compared with untreated animals at that time (Table 4).

The effect of I on ADCC activity against SRBC was next studied. ADCC activity was much enhanced by administration of I at doses of from 0.05 to 5.0 mg/kg/day (Table 5).

Effect on CFU-C Production in Culture of Mouse Bone Marrow Cells

The *in vitro* effect of I on the generation of granulocyte-macrophage progenitor cells in bone marrow was studied. The addition to the culture of from 0.001 to 0.01 μg/ml of I with LPS-induced colony-stimulating factor increased the numbers of CFU-C by about 50%. Without added colony-stimulating factor, colony formation did not occur in bone-marrow cells in either the control of I-treated cultures.

Discussion

In this study, we found that OF4949-I had antitumor activity against subcutaneous solid IMC carcinoma and was effective in protecting against pulmonary metastases of Lewis lung carcinoma. To elucidate the antitumor mechanisms of this compound, we examined the effect of I on the growth of cultured cells and on the mouse immune system. OF4949-I was not cytotoxic to any of the cells tested. In experiments that tested its effect on immune responses, I increased the numbers of PFC to SRBC slightly, suggesting that humoral immunity was not much influenced. On the other hand, I enhances the DTH response,[1] the cytostatic activity of peritoneal macrophages, and NK, and ADCC activities in mouse spleen cells. These results suggest that the antitumor effect of I could be partly explained by the augmentation of cellular immuno-response mediated by macrophages, NK cells, or both. When we compared the enhencing of macrophages, NK and ADCC, by I and by bestatin under the same experimental conditions, significant defferences were not found.

The increasing evidence that macrophages and NK cells are important in the host's defence against neoplasia has stimulated interest in agents that can activate these cells. Several natural products of small molecular weight and their derivatives, such as bestatin,[5] forphenicinol,[18] FK 156, FK 565,[19] tuftsin,[20] and myroridin-K[21] can stimulate macrophage or NK activity. These immunity-enhancing

Table 6. Effects of OF4949-I on blastogenesis of spleen cells.

OF4949-I (μg/ml)	Incorporation (cpm/culture, mean±SD)					
	C57BL/6		CDF_1			
	Without lectin	T/C (%)	Without lectin	T/C (%)	Con A (5 μg/ml)	LPS (2 μg/ml)
0 (Control)	2,155±325	100	3,645±215	100	82,296±5,228	14,879±1,473
0.1	2,984±285*	138	4,665±323*	128	84,272±9,338	14,715±1,590
1.0	3,146±225**	146	5,682±167**	156	87,564±4,324	14,475±1,463
10.0	3,392±216**	157	5,820±425**	145	81,480±7,233	14,596±2,241
100.0	ND		4,505±335*	123	85,592±6,325	14,188±1,131

ND: Not done.

Student's t-test compared with control (control, $n=6$; experimental, $n=3$).

* $P<0.05$, ** $P<0.01$.

Spleen cells of CDF_1 or C57BL/6 mice were cultured in RPMI 1640 medium with I with or without Con A (5 μg/ml) or LPS (2 μg/ml) for 3 days. Eighteen hours before being harvested, each culture was labeled with [^{3}H]TdR, and its incorporation into the cells was measured.

agents of small molecular weight, including I, may become clinically useful immuno-potentiators, because they have no antigenicity and low toxicity. Moreover, some of them enhance the production of CFU-C of bone-marrow stem cells of mice by stimuli in serum from LPS-treated mice.[5,18] In our experiment with *in vitro* culture of bone marrow cells, treatment with I also increased CFU-C formation. In a preliminary experiment, the addition of I at doses from 0.1 to 100 μg/ml enhanced incorporation of [^{3}H]thymidine into lymphocytes by about 50%, but did not influence the mitogenicity of concanavalin A (Con A) or LPS (Table 6). OF4949-I may be interesting and useful in both immunology and enzymology.[3]

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