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. They are selective inhibitors of aminopeptidase B, leucine aminopeptidase, and enkephalin-degrading aminopeptidase. These inhibitors enhance the delayed-type hypersensitivity (DTH) of mice to sheep red blood cells (SRBC), and have very low toxicity. As reported by Umezawa et al., 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.

Animals

CDF₁ mice (BALB/C×DBA/2, females, 8~15 weeks old), BDF₁ 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 CDF₁ 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 x 10⁶ cells of IMC carcinoma were implanted sc into the flank of CDF₁ mice. OF4949-I
was given ip daily for 7 days on different schedules.

To test the effect of I on secondary transplanted tumors, 2 x 10⁶ cells of IMC carcinoma were
inoculated into the left hind footpad of CDF₁ 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, 10⁵ 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 x 10⁵ viable cells) were prepared¹¹) and implanted sc in the left hind
footpad of BDF₁ mice. On day 15, the foot bearing the first tumor was surgically amputated. Meta-
static pulmonary nodules were counted on day 28. OF4949-I was given ip daily from day 1 to 11.

Antibody Formation
CDF₁ mice were immunized with 10⁶ 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 technique¹²) with SRBC and guinea pig complement.

Cytostatic Assay
Tumor-free CDF₁ 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, [³H]thymidine ([³H]TdR) was added to the culture. The cytostatic activity was as-
sayed by the inhibition of [³H]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 x 10⁶)
were transplanted sc in CDF₁ 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.

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

Cytotoxic Assay
To examine the influence of I on natural killer (NK) activity, I was injected ip into CDF₁ 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 ⁵¹Cr 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 CDF₁ 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 CDF₁ female mice were cultured in α-MEM with colony-stimulating factor
based on the method of METCALF¹⁶) and STANLEY et al.¹⁷) After 10 days of incubation, CFU-C was
counted by the method of ISHIZUKA et al.¹⁸) 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.

<table>
<thead>
<tr>
<th>OF4949-I (mg/kg/day)</th>
<th>Days -7 to -1</th>
<th>Days 0 to 6</th>
<th>Days 7 to 13</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tumor weight (mean±SD)</td>
<td>IR (%)</td>
<td>Tumor weight (mean±SD)</td>
</tr>
<tr>
<td>0 (Control)</td>
<td>4.0±2.4</td>
<td>0</td>
<td>5.7±2.3</td>
</tr>
<tr>
<td>0.05</td>
<td>4.1±1.9</td>
<td>-3</td>
<td>4.0±1.2</td>
</tr>
<tr>
<td>0.5</td>
<td>3.8±1.7</td>
<td>5</td>
<td>3.6±1.8</td>
</tr>
<tr>
<td>5</td>
<td>3.9±0.8</td>
<td>-2</td>
<td>2.1±1.0*</td>
</tr>
<tr>
<td>50</td>
<td>ND</td>
<td></td>
<td>3.1±2.0</td>
</tr>
</tbody>
</table>

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, 1×10⁶ 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).

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 cytoplastic activity of peritoneal macrophages. The intraperitoneal administration of I augmented the cytoplastic 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 \[ \text{[H]} \text{TdR} \] 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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (mg/kg)</th>
<th>% Inhibition (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E/T 50</td>
<td>E/T 25</td>
</tr>
<tr>
<td>OF4949-I</td>
<td>0 (Control)</td>
<td>51±2</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>80±6*</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>72±5*</td>
</tr>
</tbody>
</table>

\[ \text{[H]} \text{TdR} \] 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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (mg/kg)</th>
<th>% Specific lysis (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E/T 100</td>
<td>E/T 50</td>
</tr>
<tr>
<td>OF4949-Ia</td>
<td>0 (Control)</td>
<td>10.6±1.6</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>6.3±0.9</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>21.0±2.7**</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>28.1±3.4**</td>
</tr>
</tbody>
</table>

OF4949-Ib 0 (Control) 11.6±0.2 9.3±1.0 6.1±1.3 3.7±0.9
0.03 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

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.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose (mg/kg/day)</th>
<th>% Specific lysis (mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E/T 12.5</td>
<td>E/T 6.25</td>
</tr>
<tr>
<td>OF4949-Ia</td>
<td>0 (Control)</td>
<td>38.0±2.0</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>49.5±2.0*</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>46.5±3.5*</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>62.0±4.0*</td>
</tr>
</tbody>
</table>

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 or 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, 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 enhancing of macrophages, NK and ADCC, by I and by bestatin under the same experimental conditions, significant differences 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-K21) can stimulate macrophage or NK activity. These immunity-enhancing
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.\textsuperscript{5,18} In our experiment with \textit{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 \(\mu\)g/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 immuno- and enzymology.\textsuperscript{6}

### Acknowledgments

We thank Dr. JIRO IMANISHI of the Kyoto Prefectural University of Medicine, Dr. OSAMU TANABE, and co-workers of Takara Shuzo Co., Ltd., and of Nippon Shinyaku Co., Ltd., for their encouraging advice and valuable help.

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