ANTITUMOR EFFECTS OF NOVEL IMMUNOACTIVE PEPTIDES, FK-156 AND ITS SYNTHETIC DERIVATIVES

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Effects produced by intratumor or systemic application of FK-156 and its synthetic derivatives on the syngeneic P388-DBA/2 mouse system were investigated. Among 21 compounds tested, FK-156, FK-565, FR-46758, FR-48217, FR-46091 and FR-47920 substantially suppressed tumor growth when directly injected into a tumor mass and further experiments showed that FK-156, FK-565 and FR-46758 were effective even when administered subcutaneously into site remote from tumor. The mechanisms of growth inhibition are strongly suggested to be host mediated, because these three compounds have remarkably low cytotoxicity against P388 cells *in vitro*. A single dose of FK-565, however, markedly decreased body weight in healthy DBA/2 mice, whereas FK-156 and FR-46758 did not. These results indicate the superiority of FK-156 and FR-46758 as immunotherapeutic agents over FK-565 with respect to their safety for treatment of cancer. Although significant life-span prolongation could not be seen in the two-injection regimen of six compounds in either system, systemic multiple injections of FK-156 and FR-46758 provided a statistically significant increase in the median survival time of P388 tumor bearing mice.

Recently, many investigators have attempted to prove the benefits of using immunostimulants for the immunotherapeutical approaches to cancer, employing a variety of low molecular-weight synthetic or natural substances such as levamisole[1], azimexon[2], MDP[3], tuftsin[4] and bestatin[5]. Some of them are on randomized clinical trials for their efficacy and immunostimulation. Because of their chemically defined structure, these agents have the great advantage of eliminating problems associated with the use of BCG and *Corynebacterium parvum*, which are directly related to their bacterial nature.

During the course of our screening program for the discovery of novel immunostimulating agents, FK-156 was isolated from the fermentation broth of *Streptomyces olivaceogriseus* sp. nov. and *Streptomyces violaceus*[6,7,8]. Subsequently, the chemists in our laboratory succeeded in synthesizing FK-156 and a series of structurally related peptides according to methods previously reported[9]. FK-156 is a novel, low molecular-weight, acidic tetrapeptide with diverse immunostimulatory activities both *in vivo* and *in vitro*.

As shown in the preceding paper[7], FK-156 stimulates the phagocytic function of the reticuloendothelial system of animals, which is mainly responsible for the remarkable protective effect of pretreatment by FK-156 on lethal bacterial infection in rodent models. FK-156 also enhances humoral antibody production and delayed-type hyper-sensitivity reactions when administered simultaneously with antigens. These results prompted us to investigate a possible role for FK-156 as an immunotherapeutic agent.

In the previous experiments with a hepatoma AH66/Donryu rat system, there were suggestions that the administration of FK-156 before tumor inoculation resulted in an increased number of long-term survivors[7].

The present work is designed to confirm and extend these interesting observations and is further directed at screening of FK-156 and twenty of its synthetic derivatives with substantial antitumor activity. In order to examine the antitumor activity of the drugs, an *in vivo* syngeneic system of DBA/2 and P388 lymphocytic leukemia cell in solid form was employed.

Materials and Methods

Mice

Specific-pathogen-free female DBA/2 mice were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu City, Japan. They were 7~8 weeks old at the beginning of the experiments and were housed in specific-pathogen-free conditions until the experiments were terminated.

Tumor Cells

P388, a mouse lymphocytic leukemia of DBA/2 origin, was used throughout the experiments. The tumor was maintained as an ascites tumor by weekly passage of 10^6 cells in the peritoneal cavities of DBA/2 mice.

Drugs

FK-156, FK-565, FR-46758, FR-48217, FR-46091 and FR-47920 were chemically synthesized by the methods reported elsewhere[9]. FK-156, FK-565, FR-46758 and FR-48217 were dissolved in sterile physiological saline. FR-46091 and FR-47920 were dissolved in sterile saline containing 1% and 0.2% $NaHCO_3$, respectively.

Implantation and Treatment of Solid Tumor

Tumor cells aspirated from the peritoneal cavities were washed with and resuspended in serum-free RPMI-1640 medium. Only suspensions of single cells with more than 90% viability as determined by trypan blue dye exclusion were used for injection. To induce solid tumors, 10^5 viable P388 cells per 0.1 ml were injected subcutaneously into the flank of DBA/2 mice. A solid tumor was palpable by 7 days after injection. Mice inoculated with P388 tumor cells were assigned randomly into groups of 8~10 mice and were treated with drugs as follows. The drugs in 0.05 ml volumes were injected directly into a tumor mass at dose levels of 1, 10 or 100 μg/site/injection on Days 6 and 9 after tumor inoculation, or the drugs in 0.2 ml volumes were injected systemically into the nuchal area of mice at a dose level of 6 mg/kg/injection on Days 6 and 9, or 200 μg/kg/injection on Days 1, 2, 3, 4, 7, 8, 9 and 10 after tumor inoculation. Fourteen days after the inoculation of tumor cells, each tumor was removed and weighed.

Cell Growth Assay

P388 cells aspirated from the peritoneal cavities were washed with serum-free RPMI-1640 medium and adjusted to a density of 5×10^4 cells/ml in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum (Flow Laboratories) and 5×10^{-5} M 2-mercaptoethanol. One milliliter of the cell suspension was placed in 24-well flat-bottomed plastic trays and cultivated for 3 days in the presence of either 200 μl of vehicles or drugs at the indicated final concentrations at 37°C in a 95% air/5% CO_2 incubator. Cell growth and viability were examined using the trypan blue dye exclusion method.

Effect of Drugs on Body Weight Change

Groups of 10 healthy female DBA/2 mice received single subcutaneous injections of 6 mg drugs/kg and their body weights were measured for 4 successive days.

Results

Therapeutic Effects Produced by Intratumor Injection of Drugs

We used the intratumor injection system for the first screening of compounds with antitumor activity among FK-156 and twenty of its synthetic derivatives, because the therapeutic effects are considered to be quite strong[10].

Treatment with drugs was begun 7 days after tumor inoculation, because tumors were well established in almost all mice by Day 7 and therefore it was feasible to inject drugs just into a tumor mass.

In this protocol, 6 out of 21 synthetic compounds including FK-156 produced substantial antitumor efficacy. They are FK-156, FK-565, FR-46758, FR-48217, FR-46091 and FR-47920 (their chemical structures are shown in Table 1). The results of the representative experiments shown in Table 2 indicate that approximately 25~50% growth inhibition was observed by the treatment with these six compounds at dose levels of 10 or 100 μg/site, although no increase in survival time of P388 tumor bearing mice was provided in any treatment tested (data not shown).

Table 1. Chemical structures of FK-156 and its synthetic derivatives.

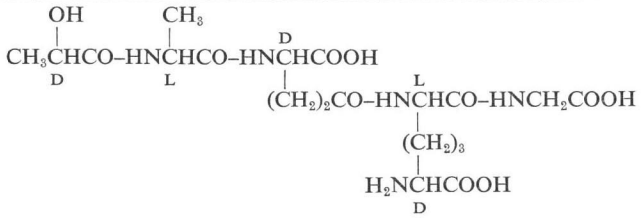
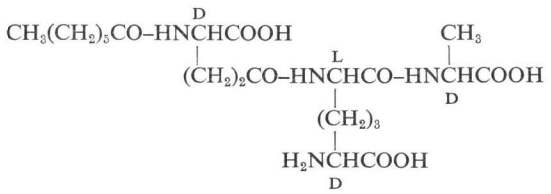
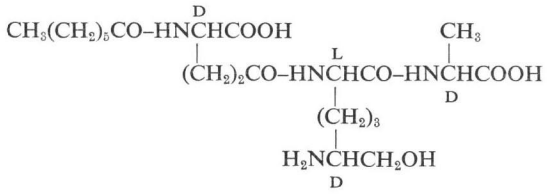
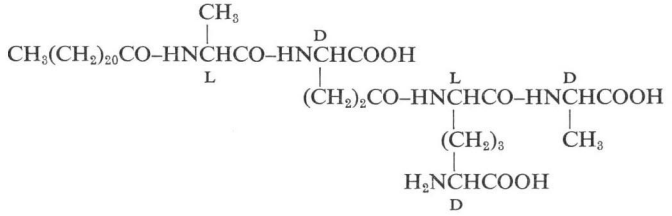
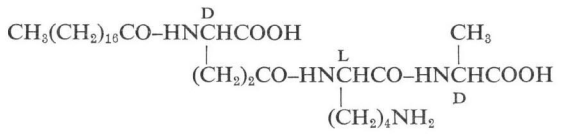
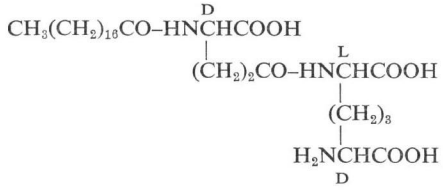
Compound	Structure
FK-156	
FK-565	
FR-46758	
FR-48217	
FR-46091	
FR-47920	

Table 2. Antitumor activity of FK-156 and its synthetic derivatives in DBA/2 mice bearing P388 solid tumor: Effects produced by intratumor injection of drugs[a].

Compound	Dose (μg/site)	Mean tumor weight at Day 14[b]	% of growth inhibition	P[c]
FK-156	Control	371±43 (231~609)	—	—
	100	188±26 (115~302)	49	<0.01
FK-565	Control	513±59 (257~703)	—	—
	10	277±43 (164~540)	46	<0.01
	100	336±35 (150~482)	35	<0.02
FR-46758	Control	248±21 (157~406)	—	—
	100	186±16 (123~273)	25	<0.05
FR-48217	Control	371±43 (231~609)	—	—
	10	251±15 (188~306)	34	<0.05
FR-46091	Control	397±35 (226~515)	—	—
	10	279±35 (97~354)	30	<0.05
	100	201±31 (87~337)	49	<0.001
FR-47920	Control	447±51 (286~718)	—	—
	1	502±65 (271~879)	−12	NS
	10	339±41 (147~513)	24	NS
	100	294±42 (145~498)	34	<0.05

a) Solid tumors were generated by subcutaneous injection of 10^5 P388 cells into the flank of DBA/2 mice on Day 0. Drugs (treated group) or vehicles (control group) in 0.05 ml volumes were injected intratumorly at dose levels of 1, 10 and 100 μg/site on Days 6 and 9 after tumor inoculation.

b) Values are mean tumor weight (mg)±SE. Numbers in parentheses, range of values.

c) Significance value as determined by Student's *t*-test. NS, not significant.

Table 3. Antitumor activity of FK-156 and its synthetic derivatives in DBA/2 mice bearing P388 solid tumor: Effects produced by systemic injection of drugs[a].

Compound	Dose (mg/kg)	Mean tumor weight at Day 14[b]	% of growth inhibition	P[c]
FK-156	Control	375±42 (199~543)	—	—
	6	270±28 (107~383)	28	<0.05
FK-565	Control	360±30 (213~538)	—	—
	6	230±17 (157~300)	36	<0.01
FR-46758	Control	282±12 (195~337)	—	—
	6	224±12 (165~335)	21	<0.02
FR-48217	Control	375±42 (199~543)	—	—
	6	257±38 (121~386)	31	<0.1
FR-46091	Control	437±43 (253~542)	—	—
	6	428±44 (276~583)	2	NS
FR-47920	Control	485±65 (257~837)	—	—
	6	387±44 (193~582)	20	NS

a) Drugs or vehicles in 0.2 ml volumes were injected subcutaneously into the nuchal area at a dose level of 6 mg/kg on Days 6 and 9 after tumor inoculation.

b) Values are mean tumor weight (mg)±SE. Numbers in parentheses, range of values.

c) Significance value as determined by Student's *t*-test.

Therapeutic Effects Produced by Systemic Administration of Drugs

For the second screening of FK-156, FK-565, FR-46758, FR-48217, FR-46091 and FR-47920, their systemic antitumor efficacy was examined on the same schedule as that of the intratumor injection system in which mice received 6 mg drugs/kg on Days 6 and 9 after tumor inoculation.

Representative results shown in Table 3 clearly demonstrate that significant therapeutical effects were achieved by treatment with FK-156, FK-565, FR-46758 ($P<0.05$) and FR-48217 ($P<0.1$), whereas

Table 4. Antitumor activity of FK-156 and its synthetic derivatives in DBA/2 mice bearing P388 solid tumor: Systemic effects in multiple-dose regimen[a].

Compound	Dose (μg/kg)	Mean tumor weight on Day 14[b]	% of growth inhibition	P[c]
FK-156	Control	270±28 (189~418)	—	—
	200	200±30 (74~351)	26	NS
FK-565	Control	311±35 (147~500)	—	—
	200	195±31 (90~367)	38	<0.05
FR-46758	Control	343±19 (227~401)	—	—
	200	309±39 (96~547)	10	NS
FR-48217	Control	477±40 (363~618)	—	—
	200	320±87 (99~743)	33	NS
FR-46091	Control	311±35 (147~500)	—	—
	200	331±52 (178~648)	−1	NS
FR-47920	Control	Not done		
	200			

a) Drugs or vehicles in 0.2 ml volumes were injected subcutaneously into the nuchal area of mice at a dose level of 200 μg/kg on Days 1,2,3,4,7,8,9 and 10 after tumor inoculation.
b) Values are mean tumor weight (mg) ±SE. Numbers in parentheses, range of values.
c) Significance value as determined by Student's *t*-test.

FR-46091 and FR-47920 failed to inhibit the growth of P388 tumor, although significant life-span prolongation was not seen in any experiments.

In another regimen in which 6 mg drugs/kg were given once on Day 6, treatment with FK-565 alone was shown to significantly suppress P388 tumor growth, but two injections of FK-565 at 200 μg/kg on Days 6 and 9 were not effective (data not shown). These results led us to investigate the therapeutic efficacy of drugs in a multiple injection regimen (200 μg/kg on Days 1, 2, 3, 4, 7, 8, 9 and 10). As shown in Table 4, only treatment with FK-565 inhibits tumor growth, but mice receiving repeated administrations of FK-565 showed considerable body weight loss. Since good tolerance was observed with FK-156 and FR-46758-treated mice, we attempted to examine the therapeutic efficacy of multiple injections of these two drugs at higher doses ranging from 200 μg/kg to 25 mg/kg.

Table 5. Effect of FK-156 and its synthetic derivatives on body weight change in healthy DBA/2 mice[a].

Compound	Body weight (g) on		P[c]
	Day 0	Day 2	
Control	20.9±0.5[b]	21.4±0.6	—
FK-156	21.0±0.3	20.9±0.4	NS
FK-565	21.2±0.4	19.7±0.5	<0.05
FR-46758	20.9±0.3	21.3±0.3	NS
FR-48217	21.3±0.4	19.4±0.5	<0.05
Control	19.4±0.2	20.0±0.3	—
FR-46091	19.8±0.2	20.4±0.3	NS
Control	20.9±0.2	20.9±0.3	—
FR-47920	21.1±0.2	21.1±0.3	NS

a) A dose of 6 mg/kg of each drug was injected subcutaneously into a group of 10 healthy DBA/2 mice. Body weights were measured on Day 0 (just before drug injection) and on Day 2.
b) Mean±SE.
c) Body weights in a drug-treated group of mice on Day 2 were compared to those in a control group and were analyzed by the Student's *t*-test. Differences in body weights between drug-treated and control groups on Day 0 were not significant.
NS, not significant.

Table 6 demonstrates that treatment with 8 injections of FK-156 at 5 mg/kg provided a considerable increase (3.9 days) in the median survival time ($P<0.01$ determined by the Mann-Whitney U-test). Treatment with FK-156 at 1 mg/kg also prolonged the median survival time with statistical significance ($P<0.1$), but that with 0.2 or 25 mg/kg was ineffective. FK-156 appeared to be well-tolerated during the course of treatment with no toxic deaths when administered at doses up to 5 mg/kg. A decrease in

Table 6. Effect of FK-156 on life-spans of mice bearing P388 tumor[a].

Treatment	Dose (mg/kg)	No. of survivors on Day														MST[b]	P[c]
		0	16	17	18	19	20	21	22	23	24	25	26	27	28		
FK-156	0	10	10	9	9	5	4	2	1	0						20.0	—
	0.2	10	10	10	9	4	3	3	2	1	0					19.4	NS
	1	10	10	10	9	9	7	6	3	1	0					22.0	<0.1
	5	10	10	10	10	10	9	8	6	6	2	1	0			23.9	<0.01
	25	10	10	10	10	6	5	4	3	3	2	1	1	1	0	21.0	NS

a) Solid tumors were generated by subcutaneous injections of 10^5 P388 cells into the flank of DBA/2 mice on Day 0 and randomly divided into 5 groups of 10 mice. Drug or sterile saline in 0.2 ml volumes were injected subcutaneously into the nuchal area of mice at the indicated dose levels on Days 1, 2, 3, 4, 7, 8, 9 and 10 after tumor inoculation.

b) Median survival time (days).

c) Significance value as determined by the Mann-Whitney U-test.
NS, not significant.

Table 7. Effect of FR-46758 on life-spans of mice bearing P388 tumor[a].

Treatment	Dose (mg/kg)	No. of survivors on Day								MST[b]	P[c]
		0	13	14	15	16	17	18	19		
FR-46758	0	10	10	9	6	3	0			16.0	—
	0.2	9	9	9	6	3	1	0		16.2	NS
	1	9	9	9	9	5	2	0		16.8	<0.1
	5	10	10	10	10	7	4	0		17.3	<0.05
	25	9	9	9	8	6	4	1	0	17.5	<0.05

a) Solid tumors were generated by subcutaneous injections of 10^5 P388 cells into the flank of DBA/2 mice on Day 0 and randomly divided into 5 groups of 10 mice. Drug or sterile saline in 0.2 ml volumes were injected subcutaneously into the nuchal area of mice at the indicated dose levels on Days 1, 2, 3, 4, 7, 8, 9 and 10 after tumor inoculation.

b) Median survival time (days).

c) Significance value as determined by the Mann-Whitney U-test.
NS, not significant.

body weight was observed in a group of mice receiving FK-156 at 25 mg/kg. On the other hand, good tolerance was observed in all groups of FR-46758-treated mice at any dosage levels tested. As shown in Table 7, treatment with 8 injections of FR-46758 at 5 and 25 mg/kg yielded a statistically significant increase in the median survival time of 1.3 and 1.5 days, respectively ($P<0.05$). The longer median survival time of mice in a control group was observed in a experiment with FK-156, which might be due to the lower viability of P388 cells used for injection (approximately 80%).

Inhibitory Effects of Drugs on P388 Cell Proliferation

Fig. 1 depicts the growth of P388 cells in liquid suspension culture in the continuous presence of drugs for 3 days. FK-156, FK-565 and FR-46758 did not inhibit the growth of P388 cells even at a concentration as high as 1 mg/ml. In contrast, complete inhibition was observed when cells were incubated with 100 μg/ml of FR-48217, FR-46091 or FR-47920. However, after 3 days of exposure to 10 μg/ml of these drugs, P388 cells grew at exactly the same rate as controls.

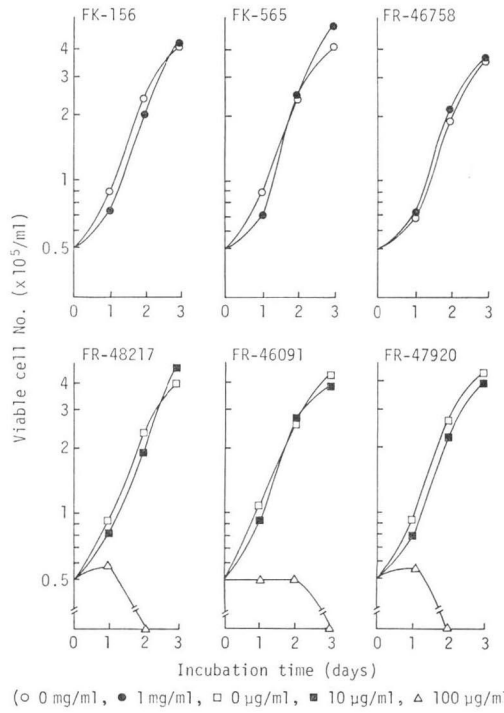
Toxic Effects of Drugs in Healthy Mice

Body weights of groups of 10 healthy DBA/2 mice were monitored for 4 successive days after single

subcutaneous injections of 6 mg drugs/kg. During the course of observation, body weights in groups of mice that received FK-156, FR-46758, FR-46091 or FR-47920 were similar to those in corresponding controls and were markedly lower in FK-565 or FR-48217-treated mice. Table 5 shows body weights of mice in control or experimental groups two days after drug injection, on which the decrease in body weight was most marked and was gradually recovered after that.

Fig. 1. *In vitro* effects of FK-156 and its synthetic derivatives on P388 cell proliferation.

These graphs dipict the growth curves of P388 cells incubated with or without drugs at the indicated concentrations. (See Materials and Methods)



Discussion

These studies were directed at screening and comparison of FK-156 and twenty of its synthetic derivatives with substantial therapeutic activity administered intratumorly or systemically on the same schedule to P388 tumor bearing mice. Our results indicate that FK-156, FK-565, FR-46758, FR-48217, FR-46091 and FR-47920 are capable of inducing antitumor activity in mice which is presented in terms of percent inhibition of tumor growth in comparison to vehicle-treated control, but all six compounds fail to prolong survival time in two-injection regimen of both treatment systems, in which approximately 50% inhibition at most was observed.

As suggested by the results shown in Tables 2 and 3, FK-156, FK-565 and FR-46758 were clearly more therapeutically effective than FR-48217, FR-46091 and FR-47920 based on inhibition of tumor growth both in intratumor and systemic injection experiments. It appears that to achieve appreciable systemic antitumor effect, FK-156, FK-565 or FR-46758 should be given at dosage levels of as high as 6 mg/kg/injection, because treatment with FK-565 at 200 μg/kg given twice was ineffective. Multiple injections of FK-565 at 200 μg/kg, however, showed significant inhibition of tumor growth, whereas the same treatment with FK-156 or FR-46758 failed. But in preliminary experiments for confirming the safety of drugs, a single dose of FK-565 (6 mg/kg) caused marked loss of body weight in healthy DBA/2 mice whereas FK-156 and FR-46758 showed almost no toxic effect (Table 5).

Taken together, these data suggest that the most potent of six compounds with substantial antitumor activity appears to be FK-565, but FK-156 and FR-46758 are far superior to FK-565 with respect to safety as an immunotherapeutic agent for treatment of cancer. In fact, treatment of mice with multiple injections of FK-156 or FR-46758 appeared to be well tolerated at dose levels up to 5 mg/kg or 25 mg/kg, respectively, and subsequently resulted in a statistically significant increase in the median survival time of P388 tumor bearing mice (Tables 6 and 7). Better tolerance was observed in FR-46758-treated mice, however, the magnitude of growth inhibition resulting from the administration of FR-46758 appeared somewhat less strong than that of FK-156. Since mice in all groups died from metastases of the primary tumor, these results suggest that the growth inhibition of the primary tumor might result in preventing the dissemination of P388 cells from the primary implant and thus be responsible for significant life-span prolongation in P388-DBA/2 mouse system.

In experiments with lethal bacterial or viral infections, pretreatment with FK-565 produced the most potent protective activity against *Escherichia coli* or Herpes simplex virus infection (in press). It is of interest that FK-565 possesses the most potent antitumor and antiinfectious activities.

For evaluation of the immunotherapeutic activity of these drugs, results obtained by systemic injection are more reliable than those by intratumor injection, because the therapeutic effects of the latter were sometimes caused by direct cytotoxicity against tumor cells. In that sense, the results obtained with FR-48217, FR-46091 and FR-47920 may be more questionable because a direct cytotoxicity of these compounds on P388 cell proliferation possibly can be responsible for the inhibition of tumor growth (Fig. 1). The failure of FR-46091 and FR-47920 to suppress tumor growth when injected systemically might support this line of speculation. FR-48217, however, had appreciable systemic antitumor activity, which suggests that the therapeutic action by FR-48217 might not be caused simply by its toxic effect.

In contrast, the therapeutic activity of FK-156, FK-565 and FR-46758 was observed both in intratumor and systemic injection systems (Tables 2 and 3), and these three compounds had very little toxicity for P388 cell (Fig. 1). These data strongly suggest that the mechanisms of antitumor action by FK-156, FK-565 and FR-46758 are host mediated. They might possibly involve the participation of macrophages or natural killer cells, since it is well known that most agents that are effective under systemic conditions of treatment are able to activate macrophages and natural killer cells[10], which subsequently exert cytotoxicity toward target tumor cells.

Recently, FLEISCHMANN *et al.* reported that treatment with combined virus-induced interferon and immune interferon preparations delayed tumor development and increased the mean survival time by 4.5 days in P388-DBA/2 mouse system[11]. In the regimen employed, mice received combined interferon preparations at the approximate site of tumor cell injection and treatment was begun 3 hours before tumor cell inoculation and continued daily for 15 days. The antitumor activity of interferon preparations was not examined in the present study. However, Nocardia CWS, which has been shown to possess antitumor and antimetastatic activities in rodent models[12,13], was ineffective in either the intratumor or systemic injection systems at the same doses as those of FK-156 and the related compounds employed in the present study (data not shown).

The antitumor activity reported in this paper is not so remarkable when compared with that of anticancer chemotherapeutic agents. However, it has been well recognized that for obtaining significant antitumor effects employing immune adjuvants, experimental conditions such as the tumor cell burden, the timing of treatment and the mode of injection appear to be crucial and therefore should be carefully chosen. We expect that a suitable experimental design in future studies would prove a series of these compounds to be useful immunomodifiers with therapeutic antitumor activity.

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