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A New Potent Angiogenesis Inhibitor, FR-118487

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A new angiogenesis inhibitor, FR-118487 was obtained by chemical modification of FR-111142 which was isolated from the fermentation products of *Scolecobasidium arenarium* F-2015. The antiangiogenic activity of FR-118487 was compared with that of the parent compound, FR-111142. In the endothelial cell proliferation test *in vitro* and the angiogenesis in the chick embryo chorioallantoic membrane assay, FR-118487 had about 5~10 times stronger antiangiogenic activities than FR-111142. In addition, FR-118487 inhibited the angiogenesis in the rabbit corneal assay and suppressed the solid tumor growth in mice. These findings showed that FR-118487 would be a unique antiangiogenic agent with promising antitumor activity.

Angiogenesis is the process of new blood vessel formation by the endothelial cells. This biological response is often associated with some diseases, such as solid tumor (3), diabetic retinopathy (4), and rheumatoid arthritis (9). Several compounds which inhibited angiogenesis, protamine (14), angiostatic steroids (4), retinoid (10) and cartilage factors (8) have been found, although they are not clinically useful for toxicity and potency. Therefore, it is an interest to find out new angiogenesis inhibitors.

During the course of a screening for new angiogenesis inhibitors from soil microorganisms, we found that a fungus *Scolecobasidium arenarium* F-2015 produced a novel angiogenesis inhibitor, FR-111142. Furthermore, this discovery of FR-111142 led us to search for derivatives with more potent antiangiogenic activity and weaker side effect than the parent compound FR-111142, and we have chemically modified and tested many derivatives of FR-111142 for cytotoxicity against endothelial cells *in vitro*. From these, we selected FR-118487.

This paper describes the taxonomy of the producing strain, isolation, physico-chemical properties and biological activities of FR-111142, and the antiangiogenic and antitumor activities of FR-118487.

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Key words: Angiogenesis inhibitor, fermentation product, FR-118487

MATERIALS AND METHODS

Culture and Medium Conditions

The microorganism used in this study, *Scolecobasidium arenarium* F-2015 was obtained from a soil. The seed medium contained soluble starch 2%, corn starch 1%, glucose 1%, cotton seed flour 1%, dried yeast 1%, peptone 0.5%, corn steep liquor 0.5% and $CaCO_3$ 0.2%, pH 6.0. The production medium contained soluble starch 3%, glucose 1%, wheat germ 1%, cotton seed meal 0.5%, $CaCO_3$ 0.2% and Adekanol (antiform) 0.025%.

A loopful of slant culture of *S. arenarium* F-2015 was inoculated to each of twenty 500-m*l* Erlenmeyer flasks containing 160 m*l* of the seed medium and cultured at 25℃ for 72 hours on a rotary shaker with 7.5-cm throw at 200 rpm. Three liters of the seed culture were inoculated to the production medium (120 liters) in a 200-liter jar fermentor, which had been sterilized at 120℃ for 30 minutes, and cultured at 25℃ for 72 hours under aeration of 120 liters/minutes and agitation of 250 rpm.

Chorioallantoic Membrane (CAM) Assay

CAM assay was carried out according to the method described by Tanaka *et al.* (13) with a slight modification. Fertilized eggs were incubated in an egg incubator at 37℃ for 5 days. Samples dissolved in the methylene chloride solution containing ethylene-vinyl acetate (EVA)

copolymer (8%, w/v), were pipetted onto Teflon rod and allowed to dry. The resultant pellets were placed on the 5-day CAMs. After the eggs were incubated at 37℃ for further 2 days, fat emulsion was injected into the 7-day chorioallantois. The antiangiogenic response was evaluated by measuring an avascular zone in the CAM around the pellet (1). The antiangiogenic response was scored qualitatively as normal, normal capillaries beneath and around the pellet; lower, area beneath the pellet is avascular, avascular, area of avascularity extends around the pellet (diameter of up to 5 mm or more).

Rabbit Corneal Assay

The rabbit corneal assay was carried out according to the method described by Gimbrone *et al.* (5). In brief, female New Zealand white rabbits (9 weeks old) were anesthetized by intravenous injection of pentobarbital supplemented with topical application of a few drops of xylocaine, an ophthalmic surface anaesthetic. The eyeball was moved forward, and a transverse 1.5 mm superficial incision was made in the corneal dome to one side of its center with the use of a blade. The cut penetrated about half way through the cornea, deep enough to permit insertion of a spatula. The pocket was prepared with malleable iris spatula. EVA pellets (about 1 mm^3) containing 300 μg of endothelial cell growth supplement (ECGS), 30 μg of heparin and/or FR-118487 (5~20 μg) were prepared and implanted in the pocket of cornea within about 1.5 mm from the limbus. The control pellets without samples were similarly implanted in the opposite eye of each rabbit. Four eyes were examined in each group. On day 10, the rabbit was anesthetized by intravenous injection of pentobarbital. The cornea was examined and photographed, and the length of new blood vessels was measured from the picture.

***In vitro* Cytotoxic Activity**

Cytotoxic activities of FR-111142 and FR-118487 were determined as follows. Concentration of the compound required for 50% inhibition of cell growth (IC_{50}, μg/ml) was examined by plotting the logarithms of the concentration versus the growth rate (percentage of control) of the treated cells. Endothelial cells from human umbilical vein (HUVEC, 2×10^3 cells/ml) were plated on microtiter plates previously coated with human fibronectin and incubated with MCDB-151 medium supplemented with 15% fetal bovine serum (FBS), 100 μg/ml ECGS, 10 μg/ml heparin, 100 units/ml penicillin and 100 μg/ml streptomycin. Murine leukemia P388, lymphoma EL-4 and fibrosarcoma Meth A cells (1×10^4 cells/ml) were maintained and treated in suspension in RPMI 1640 medium supplemented with 10% FBS, 2-mercaptoethanol (5×10^{-5} M) and the antibiotics. Baby hamster kidney (BHK) cells and murine adenocarcinoma colon 38 cells (1×10^4 cells/ml) were maintained and treated in adherence in RPMI 1640 medium supplemented with 10% FBS and the antibiotics. Endothelial cells or other cells were incubated for 120 hours or 48 hours at 37℃ in 5% CO_2 incubator, respectively. The cytotoxicity was colorimetrically determined at 540 nm after staining the viable cells with neutral red solution.

Antitumor Activity

Murine fibrosarcoma Meth A was maintained intraperitoneally by serial passage in Balb/c mice (female, 8 weeks old). Murine colon 38 carcinoma was maintained subcutaneously by serial passage in BDF1 mice (female, 8 weeks old). In the experiments, Meth A was implanted intradermally into Balb/c mice at an inoculum size of 1×10^5 cells per mouse. Fragments ($2\times2\times2$ mm) of colon 38 were implanted subcutaneously into the left flank of BDF1 mice. Twenty-four hours after the implantation of tumor cells, graded doses of FR-118487 were administered subcutaneously to mice once a day on days 1-4, 7-11 and 14-18 in the test on Meth A, or on days 8, 11, 13, 15, 20, 25 and 27 in the test on colon 38, respectively. FR-118487 was suspended in the sesame oil containing aluminium stearate (3%, w/w) and lactic acid-glycolic acid (50:50) copolymer (five times the amount of FR-118487). Control animals received subcutaneously sesame oil solution containing aluminium stearate and lactic acid-glycolic acid copolymer. Eight mice were used for each experimental group. Drug efficacy against solid tumor implanted was expressed as mean tumor weight. Tumor weight, as derived from caliper measurements of length (a) and width (b) of tumors in mm, was calculated by the following equation; tumor weight (mg)$=1/2\times a\times b2$.

Chemical Synthesis of FR-118487

Hydrolysis of FR-111142: To a solution of FR-111142 (1.23 g) in ethanol (13 ml) was added dropwisely aqueous 1 N NaOH solution (3.1 ml) at 5℃. After stirring at the same temperature for 3 hours, the mixture was concentrated *in vacuo*. The residue was partitioned between diethyl ether (60 ml) and aqueous $NaHCO_3$ solution (15 ml). The separated organic layer was dried over $MgSO_4$, and concentrated *in vacuo* to yield fumagillol (880 mg) as a solid.

Synthesis of FR-118487: To a solution (14.8 mg) in freshly distilled tetrahydrofuran (0.5 ml) was added sodium hydride (2.5 mg, 60% oil dispersion) in one portion at 5℃. The mixture was stirred for half an hour at the same temperature and then methyl isocyanate (6 μl) was added thereto. After stirring at ambient temperature for 1.5 hours, the mixture was treated with methyl isocyanate (6 μl) to complete the reaction. The reaction mixture was stirred for 3.25 hours at ambient temperature and diluted with diethyl ether. The organic layer was washed with brine, dried, and concentrated to give

a crude oil which was purified by preparative thin layer chromatography to yield FR-118487 (11 mg) as crystals (see Fig. 5).

RESULTS

Identification and Characterization of the Producing Strain of FR-111142

The strain F-2105 was originally isolated from a decaying wood debris collected at the beach of Uchinada, Ishikawa Prefecture, Japan.

This organism grew rapidly on various agar media, attaining 4 to 6 cm in diameter after one week at 25℃, and formed dark olive and felty colonies. Conidial structures were abundantly produced on the colony surface. Its conidiogenesis was probably holoblastic. Conidia were solitary, and formed sympodially on short conidiophores (Fig. 1).

The conidiophores were mononematous, pale brown, smooth, simple or branched, straight or flexuous, (8-) 12~35 (-70) μm long and 2~4 μm thick. The terminals of conidiophores were swollen, measuring 4~5 μm in diameter, cicatrized, and produced 2 to 12 conidia in cluster. The conidia were brown, obovoid or ellipsoidal to cylindrical, with a marked projection at the base, minutely but distinctly verruculose, 1-3 (-5) septate, often with dark septa and a dark spot at both ends, and (9-) 12~25 (-30)×4~7 μm. Vegetative hyphae were septate, hyaline, smooth and branched. Chlamydospores were absent.

According to the taxonomic criteria, the strain F-2015 resembled *Scolecobasidium arenarium* (Nicot) M.B. Ellis, 1976 (2), and above characeteristics correspond with this species description by Ellis. Thus, we considered this organism was one strain of *S. arenarium*. Incidentally, this species has another name *Dendryphiella arenarium* Nicot 1958 (7), base on enteroblastic, polytretic conidiogenesis. Our scanning microscopic observation showed that its conidiogenesis seemed to be holoblastic (Fig. 2), consequently, we selected the former name. However, the ultrastructure of its conidiogenesis remains to be resolved.

The strain was designated *Scolecobasidum arenarium* F-2015, and deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, as FERM BP-1520.

Isolation and Physico-Chemical Properties of FR-111142

The cultured broth (120 liters) was filtered with an aid of diatomaceous earth (20 kg). The filtrate (95 liters) was extracted with 95 liters of ethyl acetate and stirred for 10 minutes. This extraction procedure was carried out twice and the extracts were combined. The extracts were concentrated *in vacuo* to a volume of 4 liters. After dehydration with anhydrous sodium sulfate, the extract was further concentrated *in vacuo* to dryness. The oily materials obtained were mixed with silica gel (100 g) and applied to a 1-liter silica gel chromatography column. After washing with 3 liers of n-hexane and then 3 liters of a mixture of n-hexane-ethyl acetate (1:1), the column was eluted with 4 liters of ethyl acetate. The eluates were concentrated *in vacuo* to dryness. The active materials were mixed with silica gel and applied to a silica gel column (400 ml). After washing with 1.2 liters of chloroform and a mixture of chloroform-methanol (100:1), the column was eluted with 1.2 liters of a mixture of chloroform-methanol (75:1 and 50:1), stepwisely. The active fractions eluted were concentrated *in*

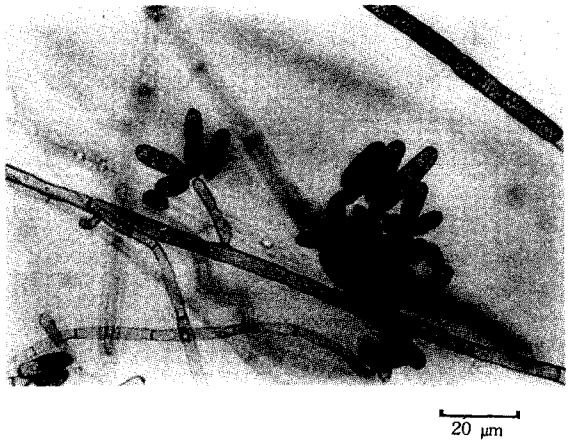


Fig. 1. Monograph of conidial structures of *S. arenarium* F-2015.

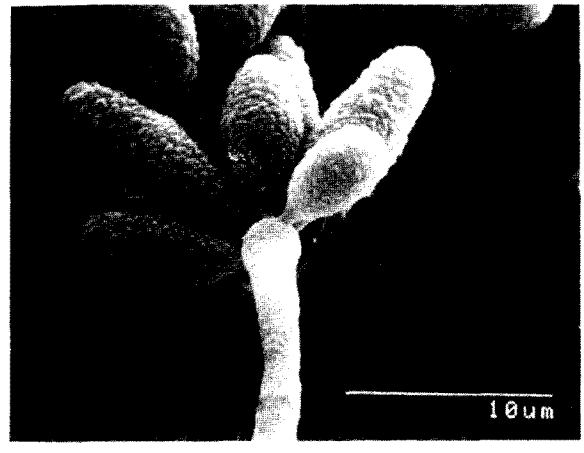


Fig. 2. Scanning electron micrograph of *S. arenarium* F-2015.

vacuo to a volume of 5 ml. The concentrate was mixed with octadecyl-substituted silica gel (ODS) and subjected to chromatography on ODS gel (100 ml). The column was washed with a mixture of methanol-H_2O (100:1) and eluted with 300 ml of a mixture of methanol-H_2O (3:2). The active fractions were collected and concentrated *in vacuo* to give a purified colorless powder of FR-111142 (100 mg).

FR-111142 was purified from the culture broth of *S. arenarium* F-2015 by measuring its inhibitory activity of proliferation of endothelial cells.

FR-111142 was readily soluble in methanol and acetone, and insoluble in water and n-hexane. FR-111142 gave positive raction to celium sulfate and iodine vapor, though negative to Molish, Ninhydrin and ferric chloride reagents.

Its purity was confirmed by ^1H NMR (Fig. 3) and mass spectrum. The physico-chemical properties of FR-111 142 are summarized in Table 1. From the data of the ^{13}C NMR (Fig. 4) and FAB-MS spectra, together with the elemental analysis, the molecular formula of FR-111142 was determined to be $C_{22}H_{34}O_7$. The structure of FR-111142 was supposed to be (3R,4S,5S,6R)-5-methoxy-4-[(2R,3R)-2-methyl-3-(3-methlyl-2-butenyl)oxiranyl]-1-oxaspiro[2.5]oct-6-yl(E)-4,5-dihydroxy-2-hexenoate (Fig. 5) on the basis of chemical and spectroscopic evidence. Full account of structural studies on FR-11 1142 will published elsewhere (12).

Cytotoxicity against *in vitro* Cultured Cells

The cytotoxic effects of FR-111142 and FR-118487 were examined against *in vitro* cultured cells. The results

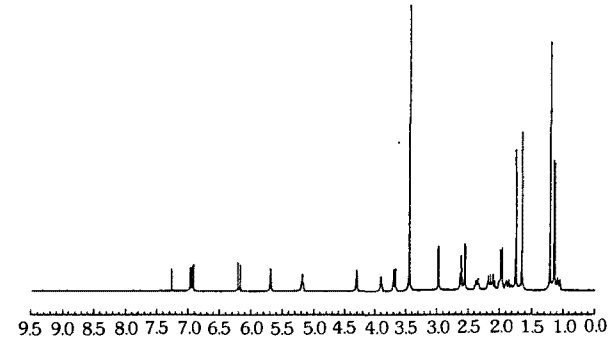


Fig. 3. 400 MHz ^1H-NMR spectrum of FR-111142.

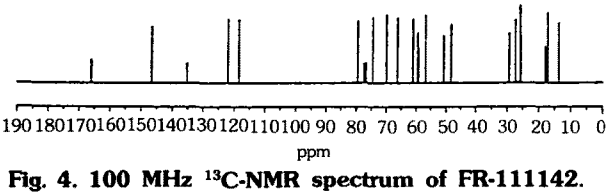


Fig. 4. 100 MHz ^{13}C-NMR spectrum of FR-111142.

Table 1. Physico-chemical properties of FR-111142

Appearance	White powder
Molecular formula	$C_{22}H_{34}O_7$
Molecular weight (m/z)	410
Elementary analysis	
Calcd. for $C_{22}H_{34}O_7$	C 64.47, H 8.35
Found	C 64.55, H 8.86
FAB-MS	411 $(M+H)^+$
$[\alpha]_D^{25}$	−52° (c1.0, MeOH)
UV	End absorption (in $CHCl_3$)
IR $\nu_{max}^{CHCl_3}$ (cm^{-1})	3450, 22960, 2920, 1710, 1650, 1440, 1370, 1300, 1260, 1200, 1170, 1120, 1100, 1070, 1050, 1000, 980, 920, 880, 830
TLC	
(silica gel plate) Rf[a]	0.35
Rf[b]	0.39
(ODS gel plate) Rf[c]	0.44

[a] Solvent system; Chloroform-methanol, 10:1
[b] Solvent system; Benzene-ethyl acetate-ethanol, 6:3:1
[c] Solvent system; Methanol-water, 75:25

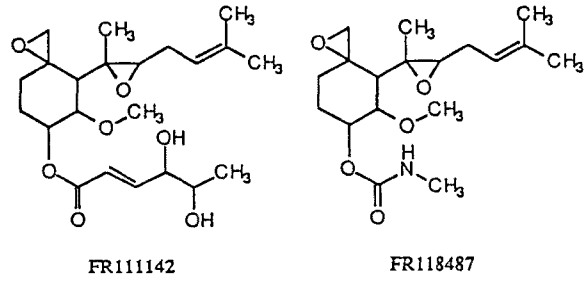


Fig. 5. Chemical structures of FR-111142 and FR-118487.

are shown in Table 2. FR-111142 and FR-118487 were effective against HUVEC cells and EL-4 cells at low concentrations, though these effects were cytostatic. Further, the cytotoxicity of FR-118487 was five times stronger than that of FR-111142 against HUVEC cells.

Effect of FR-111142 and FR-118487 on Angiogenesis in Chick CAM

The inhibitory effects of FR-111142 and FR-118487 on angiogenesis in chick CAM were examined. At least 15 eggs were used for each dose of these compounds. The results are shown in Table 3. These compounds produced an avascular zone in the CAM in a dose dependent manner (Fig. 6). Compared to the empty pellet without FR-111142 or FR-118487, FR-111142 at doses of 5.0~430 μg/pellet and FR-118487 at doses of 0.5~320 μg/pellet displayed potent antiangiogenic activity in this system. Inhibitory effect of FR-118487 against

Table 2. Inhibitory effects of FR-111142 and FR-118487 on angiogenesis in CAMs

Dose (μg/pellet)	Number of eggs with following capillary density*					
	FR-111142			FR-118487		
	Normal	Lower	Avascular	Normal	Lower	Avascular
0	20	0	0	30	0	0
0.32				10	3	2
0.5				7	3	6
1.0	13	2	0	6	4	5
3.2	13	3	1	7	5	6
5.0	7	4	5			
10	5	5	8	5	5	8
32				5	5	10
50	4	5	9			
100	3	3	10	2	7	19
320	0	4	11	0	5	23

*Density of capillaries developed around the pellet.

Table 3. Cytotoxicity of FR-111142 and FR-118487 on various cells

Cell line	MIC (μg/ml)	
	FR-111142	FR-118487
Human umbilical vein endothelial cell	5.0×10^{4}	1.0×10^{4}
P388 leukemia cell	42	30
Meth A fibrosarcoma cell	25	60
Colon 38 adenocarcinoma cell	42	60
EL-4 lymphoma cell	3.8×10^{4}	1.0×10^{4}
BHK kidney cell	10	15

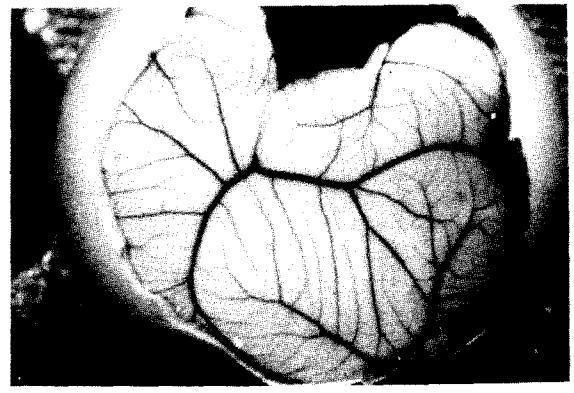
angiogenesis on CAM was ten times stronger than that of FR-111142. In addition, these compound apparently caused no damage to the growth of embryo.

Effect of FR-118487 on Angiogenesis in Rabbit Cornea

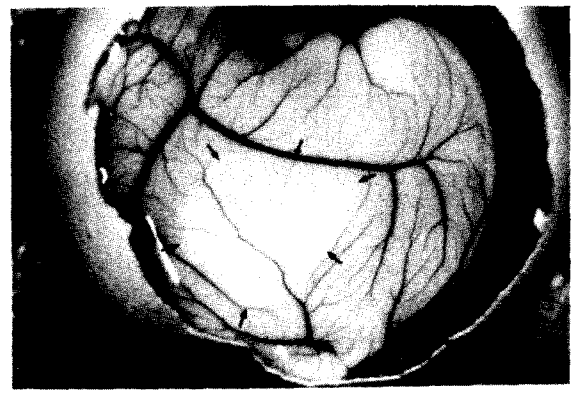
Further the inhibitory effect of FR-118487 on the angiogenesis induced by an angiogenic factor in rabbit cornea was tested. Five days after implantation, corneas were penetrated by new capillaries which grew from the limbal blood vessels. Angiogenic activity was evaluated by new blood vessels reaching the sample pellet. Compared to the empty pellet without FR-118487 (control), FR-118487 at doses of 100~500 μg/pellet remarkably suppressed capillary growth (Table 4). The similar inhibitory effect of FR-118487 on angiogenesis in rabbit cornea was obtained with systemic administration of FR-118487 (unpublished data).

Antitumor Activity of FR-118487

The *in vivo* antitumor activity of FR-118487 was determined in experimental tumor system in mice. The results are shown in Fig. 7. FR-118487 was quite effective against Meth A and colon 38 at doses of 0.3~5 mg/kg. Furthermore, when FR-118487 was administered subcutaneously to mice, it produced slight weight loss at high dose (about 10% body weight of control



(A)



(B)

Fig. 6. Effect of FR-111142 on embryonic angiogenesis. EVA copolymer pellets containing FR-111142 ((A) 0 μg, (B) 200 μg) were placed on the CAMs of 5-day eggs. After 2 days, the angiogenesis inhibitory activity was determined, ×2.25.

Table 4. Inhibitory effect of FR-118487 on angiogenesis in rabbit cornea

Dose (μg/pellet)	Length of capillary growth (mm)[a] Mean±S.E.
0	1.75±0.11
20	1.34±0.14
100	0.90±0.10[b]
500	0.54±0.07[b]

[a]Value represents mean of length of each longest capillary induced in 4 eyes.

[b]Significantly different from control (0 μg/pellet) at $P<0.01$ (Student's test).

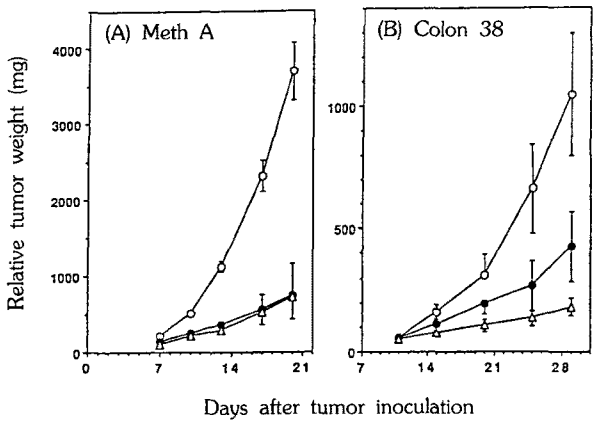


Fig. 7. Antitumor effect of FR-118487.
(A) Meth A fibrosarcoma; (B) colon 38 carcinoma. Symbols: (A) −○−, control; −●−, FR-118487, 0.3 mg/kg; −△−, FR-118487, 1 mg/kg. (B) −○−, control, −●−, FR-118487, 1 mg/kg; −△−, FR-118487, 3 mg/kg.

mice).

Acute Toxicity

The acute toxicity of FR-111142 was determined in ddY mice (5 weeks old, female) by a single intraperitoneal or intravenous injection of graded doses of FR-118487 into 5 mice. The LD_{50} was over 1 g/kg.

DISCUSSION

FR-111142, which exhibited *in vitro* and *in vivo* angiogenesis inhibitory activity, was isolated from *Scolecobasidum arenarium* F-2015. From the evidence of physico-chemical data, FR-111142 was classified as being of the fumagillin type. Recently it was reported by Otsuka *et al.* (11) and Ingber *et al.* (6) that fumagillin was an angiogenesis inhibitor, but caused severe weight loss and was not so effective against solid tumors in mice. On the other hand, we have found that FR-11 1142 inhibited tumor growth in mice with relative few side effects (12). Further, we searched for chemically synthesized derivatives with stronger antiangiogenic activity than the parent compound, FR-111142, and selected FR-118487. FR-118487 had more potent antiangiogenic effects than FR-111142 against *in vitro* cultured HUVEC cells and *in vivo* chick CAM assay. In addition, the present study demonstrated that FR-118487 inhibited the angiogenesis in the rat corneal assay. FR-118487 also suppressed the growth of solid tumors, Meth A and colon 38, but not active against leukemia P388.

In summary, FR-118487 has stronger antiangiogenic activity and more potent cancer chemotherapeutic effect than FR-111142. These fact suggests that FR-118487 will be a beneficial drug for the angiogenesis-dependent diseases besides solid tumors.

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