A NEW ANGIOGENESIS INHIBITOR, FR-111142

TAKANAO OTSUKA, TOSHIHIRO SHIBATA, YASUHISA TSURUMI, SHIGEHIRO TAKASE, MASAKUNI OKUHARA, HIROSHI TERANO, MASANOBU KOHSAKA and HIROSHI IMANAKA

Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 5-2-3 Tokodai, Tsukuba, Ibaraki 300-26, Japan

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FR-111142 is a new angiogenesis inhibitor produced by a fungus *Scolecobasidium arenarium* F-2015.

FR-111142 inhibited endothelial cell proliferation *in vitro* and angiogenesis in the growing chick chorioallantoic membrane model *in vivo*. Further, FR-111142 also suppressed the solid tumor growth in mice.

Angiogenesis is the process of new blood vessel formation by the endothelial cells and often associated with diseases, such as solid tumor[1], diabetic retinopathy[2], and rheumatoid arthritis[3]. Several compounds which inhibited angiogenesis, protamine[4], angiostatic steroids[2], retinoid[5] and cartilage factors[6] have been discovered, although they are not clinically useful for toxicity and potency. Therefore, it is an interest to look for new angiogenesis inhibitors.

In the course of searching for angiogenesis inhibitors from soil microorganisms, we found that a fungus *Scolecobasidium arenarium* F-2015 produced a novel angiogenesis inhibitor, FR-111142.

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

Identification of the Producing Strain

The strain F-2015 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°C, 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.

The conidiophores were mononematous, pale brown, smooth, simple or branched, straight or flexuous, $(8\sim)12\sim35(\sim70)\,\mu$m long and $2\sim4\,\mu$m thick. The terminals of conidiophores were swollen, measuring $4\sim5\,\mu$m 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, $1\sim3(\sim5)$ septate, often with dark septa and a dark spot at both ends, and $(9\sim)12\sim25(\sim30)\times4\sim7\,\mu$m. 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[7], and above characteristics 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[8], based on enteroblastic, polytretic conidiogenesis. Our scanning microscopic observation showed that its conidiogenesis seemed to be holoblastic, consequently, we selected

the former name. However, the ultrastructure of its conidiogenesis remains to be resolved.

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

Fermentation and Assay

A loopful of mature slant culture of *Scolecobasidium arenarium* F-2015, was inoculated to a seed medium (160 ml) containing soluble starch 2%, corn starch 1%, glucose 1%, cotton seed flour 1%, dried yeast 1%, peptone 0.5%, corn steep liquor 0.5% and calcium carbonate 0.2% (pH 6.0) in a 500-ml Erlenmeyer flasks and cultured at 25°C for 72 hours on a rotary shaker with 7.5 cm throw at 200 rpm.

Thirty hundred ml of the seed culture was inoculated to the production medium (120 liters) containing soluble starch 3%, glucose 1%, wheat germ 1%, cotton seed meal 0.5% and calcium carbonate 0.2% in a 200-liter jar fermenter and cultured at 25°C for 72 hours under aeration of 120 liters/minute and agitation of 250 rpm.

The production of FR-111142 in the fermentation broth of *Scolecobasidium arenarium* F-2015 was assayed by HPLC using a LiChrospher RP-18 column (4×250 mm). The solvent system was acetonitrile - methanol - H_2O (20 : 35 : 45) and the flow rate was 1.0 ml/minute. The detector wavelength was set at 210 nm. The culture broth was centrifuged at 2,000 rpm for 15 minutes. Ten μl of the supernatant was used for HPLC analysis. FR-111142 was eluted at 13.66 minutes. Production of FR-111142 reached a level of 5.1 μg/ml at 72 hours of fermentation.

Isolation and Purification

The cultured broth (120 liters) was filtered with an aid of diatomaceous earth (20 kg). The filterate (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 liters 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 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 vacuo* to a volume of 5 ml. The concentrate was mixed with a octadecyl-substituted silica gel (ODS) and subjected to chromatography on a 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).

The determination of FR-111142 at each purification step was carried out by the HPLC method as described above.

Physico-chemical Properties and Structure Elucidation

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

Fig. 1. 400 MHz ^{1}H NMR spectrum of FR-111142 in $CDCl_3$.

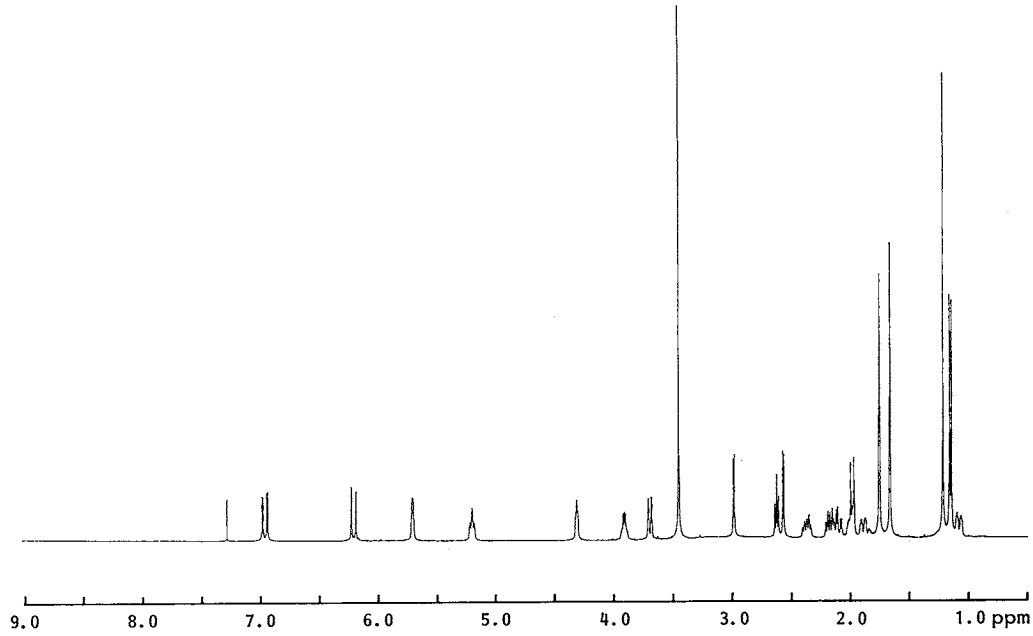
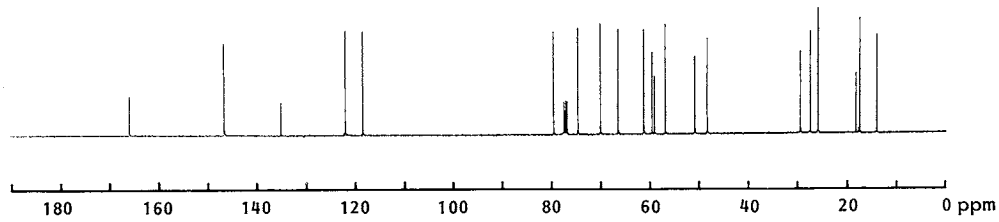


Fig. 2. 100 MHz ^{13}C NMR spectrum of FR-111142 in $CDCl_3$.



The ^{1}H and ^{13}C NMR spectra of FR-111142 are shown in Figs. 1 and 2, respectively. The other physico-chemical properties are summarized in Table 1. Fast atom bombardment (FAB) mass analysis yielded molecular formula of $C_{22}H_{34}O_7$ for FR-111142, which was consistent with elementary analysis. Base treatment of FR-111142 (**1**) (1 N NaOH, refluxed for 1 hour) gave fumagillol (**2**) which was directly identified with authentic specimen (Fig. 3). Extensive spectral analysis of ^{1}H-^{1}H COSY and ^{13}C-^{1}H COSY of **1** revealed that the fumagillol nucleus was acylated with 4,5-dihydroxy-2-hexenoyl moiety.

The ^{1}H and ^{13}C NMR data of the fatty acid portion in FR-111142 are presented in Fig. 4. The polarized olefin protons (δ_H 6.20 and 6.95) in conjunction with up-field shifted ester ^{13}C signal at δ 165.8 suggested the presence of α,β-unsaturated ester function, which was further supported by IR absorption at 1710 cm^{-1}. In the ^{1}H-^{1}H COSY spectra of **1**, the olefin proton 3′-H was coupled to 4′-H, 4′-H to 5′-H while 5′-H to 6′-CH_3. The magnitude of vicinal coupling constant (15.5 Hz) of the olefin protons defined (*E*)-geometry of the double bond. The fatty acid was esterified at C-6 because of low field ^{1}H chemical shift of 6-H (δ 5.70).

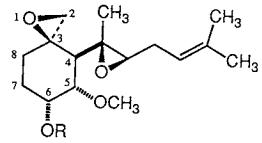
Thus, the structure of FR-111142 was elucidated to be (3*R*,4*S*,5*S*,6*R*)-5-methoxy-4-[(2*R*,3*R*)-2-methyl-3-(3-methyl-2-butenyl)oxiranyl]-1-oxaspiro[2.5]oct-6-yl(*E*)-4,5-dihydroxy-2-hexenoate. The absolute

Table 1. Physico-chemical properties of FR-111142.

Appearance		White powder
Molecular formula		$C_{22}H_{34}O_7$
Elementary analysis		
Calcd for $C_{22}H_{34}O_7$		C 64.37, H 8.35
Found		C 64.55, H 8.86
FAB-MS		411 $(M+H)^+$
$[\alpha]_D^{25}$		$-52°$ (c 1.0, MeOH)
UV		End absorption (in $CHCl_3$)
IR $\nu_{max}^{CHCl_3}$ cm^{-1}		3450, 2960, 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).

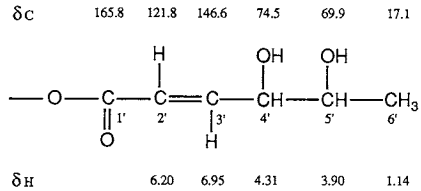
Fig. 3. Structures of FR-111142 (**1**) and fumagillol (**2**).



1 R = $-COC(H)=CCH(OH)CH(OH)CH_3$

2 R = H

Fig. 4. 4,5-Dihydroxy-2-hexanoyl portion in FR-111142 (**1**).



stereochemistry at two hydroxymethine on the fatty acid portion remains to be established.

Biological Activity

Antiangiogenic Activity

The effect of FR-111142 on angiogenesis in chick embryo chorioallantoic membrane (CAM) was examined by the method of TANAKA *et al.*[9]. In brief, fertilized eggs were incubated in an egg incubator at 37°C for 5 days. Samples dissolved in methylene chloride solution of 8% ethylene-vinyl acetate (EVA) copolymer were pipetted onto Teflon rod and allowed to dry. The resultant pellets were placed on the 5-day CAMs. At least, 15 eggs were used for each dose of FR-111142. After the eggs were incubated at 37°C for 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 according to the method of CRUM *et al.*[10] with a slight modification, followed by taking photographs of CAMs. The angiogenic response was scored qualitatively at 48 hours 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). Medroxyprogesteron acetate was used as a reference compound[11].

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

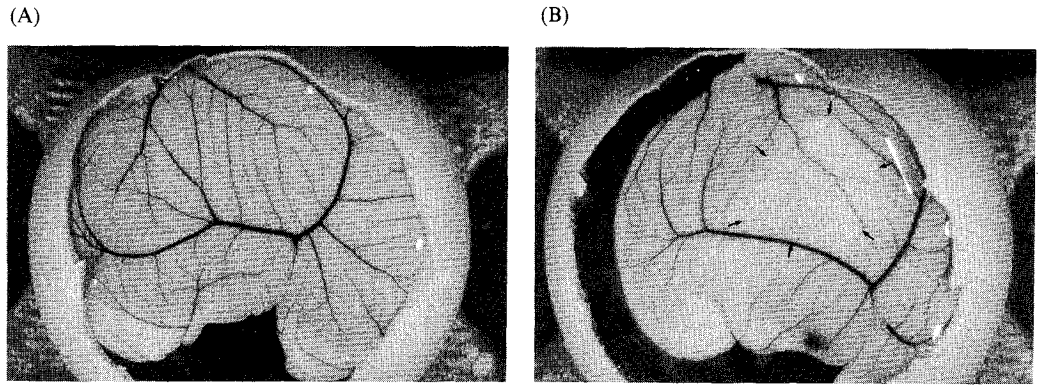
Dose (μg/pellet)	Number of CAM assayed	Number of CAM with following capillary density[a]		
		Normal	Lower	Avascular
FR-111142				
0	20	20	0	0
1.0	15	13	2	0
3.2	17	13	3	1
5.0	16	7	4	5
10	18	5	5	8
50	18	4	5	9
100	16	3	3	10
320	15	0	4	11
Medroxyprogesterone acetate				
100	31	17	8	6

[a] Density of capillaries developed around the pellet.

The results were shown in Table 2 and Fig. 5. Compared to the empty pellet without FR-111142, FR-111142 at doses of 10~320 μg/pellet displayed potent antiangiogenic activity in this system. In addition, FR-111142 apparently caused no damage to the growth of embryo.

Fig. 5. Effect of FR-111142 on embryonic angiogenesis.

Ethylene-vinyl acetate 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.



Antimicrobial Activity

Antimicrobial activity of FR-111142 was derermined by a serial broth dilution method in Nutrient broth for Gram-positive and Gram-negative bacteria and in Sabouraud media for fungi and yeast. The inoculum size was adjusted to 5×10^5 cfu/ml for bacteria and 1×10^6 cfu/ml for fungi and yeast. Minimum inhibitory concentration (MIC) is expressed in terms of μg/ml after 18 hours at 37°C for bacteria and 48~72 hours incubation at 28°C for fungi and yeast.

FR-111142 was devoid of antimicrobial activity when tested vs. the following microorganisms at 100 μg/ml; *Escherichia coli* NIHJ JC-2, *Pseudomonas aeruginosa* ATCC 14970, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* 209P, *Candida albicans*, *Aureobasidium pullulans* and *Aspergillus niger*.

Cytotoxic Activity

Cytotoxic activity of FR-111142 *in vitro* was 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 vs. the growth rate (percentage of control) of the treated cells. Fumagillin was used as a reference compound.

Endothelial cells from human umbilical vein (HUVEC, 2×10^4 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 Endothelial Cell Growth Supplement (ECGS), 10 μg/ml heparin, 50 μg/ml benzylpenicillin and 100 μg/ml streptomycin. Murine leukemia P388 cells, fibrosarcoma Meth A cells or lymphoma EL-4 cells (1×10^4 cells/ml) were maintained and treated in suspension in RPMI medium supplemented with 10% FBS, 2-mercaptoethanol (5×10^{-5} M) and the antibiotics. Baby hamster kidney (BHK) cells, murine adenocarcinoma colon 38 cells (1×10^4 cells/ml) were maintained and treated in adherence in RPMI medium supplemented with 10% FBS and the antibiotics. Endothelial cells or other cells were incubated for 120 hours or 48 hours at 37°C in 5% CO_2 incubator, respectively. The cytotoxicity was colorimetrically determined at 540 nm after staining the variable cells with neutral red solution.

The result was shown in Table 3. FR-111142 and fumagillin were effective against HUVEC cell and EL-4 cell at low concentrations, though these effects were cytostatic.

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

Cell lines	IC_{50} (μg/ml)	
	FR-111142	Fumagillin
Human umbilical vein endothelial cell	5.0×10^{-4}	1.4×10^{-3}
P388 leukemia cell	42	22
Meth A fibrosarcoma cell	25	13
Colon 38 adenocarcinoma cell	42	
EL-4 lymphoma cell	3.8×10^{-4}	1.2×10^{-3}
BHK-21 kidney cell	10	13

Table 4. Effect of FR-111142 on growth of Meth A fibrosarcoma.

Drug (mg/kg/day)		Tumor weight (mg)[a]
Control		1,198.3±132.6
Vehicle (saline)		1,348.2+125.1
FR-111142	30	117.7± 50.5
	10	240.1± 29.6
	3.3	468.6± 93.5
	1.0	682.2± 98.7
	0.3	845.3±171.8

[a] Mean±S.E.

Antitumor Activity

The *in vivo* antitumor activity of FR-111142 was determined in experimental tumor system in mice.

Mouse fibrosarcoma Meth A was maintained intraperitoneally by serial passage in Balb/c mice (female, 8 weeks old). Meth A was implanted intradermally into Balb/c mice at an inoculum size of 1×10^5 cells per mouse. Twenty-four hours after the implantation of tumor cells, graded doses of FR-111142 were administered to mice intravenously. Treatments were on day 1, 2, 3, 4 and 7, 8, 9, 10 (qd 1~4 and 7~10). FR-111142 was solubilized in methanol, concentrated *in vacuo* and then suspended in the physiological saline solution. Control animals received intravenous doses of physiological saline solution. Eight mice were used for each experimental group. Tumor weight at 14 days after the inoculation as derived from caliper measurements of the length and width of tumors, was calculated by the formula[12]: Tumor weight $(mg) = 1/2 \times a \times b^2$, where a represents the length and b represents the width (mm).

As shown in Table 4, FR-111142 was quite active against Meth A. Doses between 3.3~30 mg/kg resulted in the significant suppression of tumor growth. Further, in this study FR-111142 did not cause weight loss. FR-111142 also inhibited growth of solid tumor colon 38, but was not active against leukemia P388 (unpublished data).

In the case of fumagillin, it was required for an intraperitoneal administration at 10 mg/kg to induce 25% inhibition of Meth A tumor growth against FR-111142, 10 mg/kg, 60% inhibition. Furthermore, at the dose of 10 mg/kg fumagillin produced severe body weight loss (about 5 g), although FR-111142 did not cause the weight loss below control. These experiments were carried out according to the procedure described above with a minor modification using the intraperitoneal injection.

Acute Toxicity

The acute toxicity of FR-111142 was determined in *dd*Y mice (female, 5 weeks old) by a single intraperitoneal or intravenous injection of graded doses of FR-111142 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 *Scolecobasidium arenarium* F-2015. From the evidence of physico-chemical data, FR-111142 was classified as being of the fumagillin type. Recently, we showed that fumagillin was a potent angiogenesis inhibitor[13]. Mice treated with intraperitoneal administration of FR-111142 at 10 mg/kg daily for two weeks gained weight, whereas fumagillin caused severe weight loss, and antitumor activity of FR-111142 was more potent than that of fumagillin. Similar results have been obtained by INGBER *et al.*[14]. They have reported

that fumagillin inhibited endothelial cell proliferation *in vitro* and tumor growth *in vivo*, but the prolonged administration was limited because it caused severe weight loss.

The present data demonstrated that FR-111142 inhibited the growth of endothelial cells. Although FR-111142 caused cytostatic inhibition of endothelial cells at low concentration (5×10^{-4} μg/ml), cytotoxicity was only observed at higher concentration (50 μg/ml). One of the possibilities is the competition of FR-111142 with endothelial cell growth factor (ECGF). FR-111142, however, did not interfere with the binding of ECGF to its receptor (unpublished data). In addition, sensitivities of other cell lines were varied. FR-111142 potently inhibited the growth not only of endothelial cells but of some other cell lines *e.g.* murine lymphoma EL-4 cells. The mechanism by which FR-111142 inhibits endothelial cell growth is unresolved at the present time.

FR-111142 suppressed the solid tumor growth. On the other hand, FR-111142 inhibited the growth of such tumor cells much less than endothelial cells *in vitro*. FR-111142 was not effective against murine leukemia P388 in ascite form whose growth does not require angiogenesis. In addition, FR-111142 inhibited angiogenesis in CAM. Therefore, the potent antitumor acitivity of FR-111142 may be due to its antiangiogenic action, possibly by the inhibition of endothelial cell growth.

Considering these data, FR-111142 seems to be superior to fumagillin both in efficacy and toxicity. Further, these observations suggest that FR-111142 will be a beneficial drug for the angiogenesis dependent diseases besides solid tumors, such as diabetic retinopathy and rheumatoid arthritis.

References

1) FOLKMAN, J.: Tumor angiogenesis. Adv. Cancer Res. 43: 175~203, 1985
2) FOLKMAN, J. & D. E. INGBER: Angiostatic steroids. Method of discovery and mechanism action. Ann. Surg. 206: 374~383, 1987
3) MATSUBARA, T. & M. ZIFF: Inhibition of human endothelial cell proliferation by gold compounds. J. Clin. Invest. 79: 1440~1446, 1987
4) TAYLOR, S. & J. FOLKMAN: Protamine is an inhibitor of angiogenesis. Nature 297: 307~312, 1982
5) OIKAWA, T.; K. HIROTANI, O. NAKAMURA, K. SHUDO, A. HIRAGUN & T. IWAGUCHI: A highly potent antiangiogenic activity of retinoids. Cancer Letters 48: 157~162, 1989
6) LANGER, R.; H. BREM, K. FALTERMAN, M. KLEIN & J. FOLKMAN: Isolation of a cartilage factor that inhibits tumor neovascularization. Science 193: 70~72, 1976
7) ELLIS, M. B.: More Dematiaceous Hyphomycetes. pp. 194~195, Commonwealth Mycological Institute, Kew, 1976
8) KOHLMEYER, J. & E. KOHLMEYER: Marine Mycology—The Higher Fungi. pp. 486~490, Academic Press, 1979
9) TANAKA, N. G.; N. SAKAMOTO, A. TOHGO, Y. NISHIMURA & H. OGAWA: Inhibitory effects of anti-angiogenic agents on neovascularization and growth of the chlorioallantoic membrane (CAM).—The possibility of a new CAM assay for angiogenesis inhibition. Exp. Pathol. 30: 143~150, 1986
10) CRUM, R.; S. SZABO & J. FOLKMAN: A new class of steroids inhibits angiogenesis in the presence of heparin or a heparin fragment. Science 230: 1375~1378, 1985
11) OIKAWA, T.; K. HIROTANI, M. SHIMAMURA, H. ASHINO-Fuse & T. IWAGUCHI: Powerful antiangiogenic acitivity of herbimycin A (named angiostatic antibiotic). J. Antibiotics 42: 1202~1204, 1989
12) SHIMOMURA, K.; T. MANDA, S. MUKUMOTO, K. MASUDA, T. NAKAMURA, T. MIZOTA, S. MATSUMOTO, F. NISHIGAKI, T. OKU, J. MORI & F. SHIBAYAMA: Antitumor activity and hematotoxicity of a new, substituted dihydrobenzoxazine, FK973, in mice. Cancer Res. 11: 1166~1172, 1988
13) OTSUKA, T.; T. SHIBATA, H. TERANO, Y. TSURUMI & M. OKUHARA (Fujisawa): Fumagillin with angiogenesis inhibitory activity. Jpn. Kokai 286617 ('90), Oct. 28, 1990
14) INGBER, D.; T. FUJITA, S. KISHIMOTO, K. SUDO, T. KANAMARU, H. BREM & J. FOLKMAN: Synthetic analogues of fumagillin that inhibit angiogenesis and suppress tumor growth. Nature 348: 555~557, 1990