A New Antimitotic Substance, FR182877

I. Taxonomy, Fermentation, Isolation, Physico-chemical Properties and Biological Activities

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Using the characteristic morphological changes of mammalian cells, we screened novel antimitotic substances and found that a strain of *Streptomyces* sp. No.9885 produced FR182877. This substance was isolated from the culture broth by ethyl acetate extraction, silica gel column chromatography and ODS column chromatography. Structural studies on FR182877 suggested that it had a unique hexacyclic structure encompassing its highly strained double bond. FR182877 exhibited potent antitumor activities against murine ascitic tumor and solid tumor *in vivo*.

The antimitotic drugs, such as *vinca* alkaloids or taxol, are widely used in the chemotherapy of human cancer[1,2]. These drugs inhibit the cell cycle transition at M phase and are inhibitors of microtubule assembly or disassembly in eukaryotic cells[3,4]. In view of the clinical significance of antimitotic drugs and also because microtubule is one of the fundamental components of cytoskeleton of eukaryotic cells, it might be a principal target site in a screen for more effective antitumor drugs.

In addition, in the course of basic biological experiments using antimitotic drugs in clinical use, we found that the mammalian cells, baby hamster kidney (BHK) cells, were transformed to an amoebiform state and further formed a multinuclear state, when exposed to antimitotic drugs at the concentration in the vicinity of their IC_{50}.

Using these characteristic morphological changes of BHK cells, we attempted to screen new antimitotic substances from microbial products. As a result, we isolated a new antimitotic substance FR182877 from the culture broth of *Streptomyces* sp. No. 9885[5]. This paper describes the taxonomy of the producing strain, isolation, physico-chemical properties and biological activities of this substance.

Materials and Methods

Microorganism and Taxonomy

Strain No. 9885 was isolated from a soil sample collected at Tokushima Prefecture, Japan. For the taxonomic study of strain No. 9885, the methods and media described by SHIRLING and GOTTLIEB[6] and by WAKSMAN[7] were employed. The observations were made after 21 days cultivation at 30°C. The morphological observations were made on the culture grown on yeast extract - malt extract agar, oat-meal agar and yeast extract - starch - glucose agar containing yeast extract 0.2%, soluble starch 1%, glucose 0.5% and agar 1.6% (adjusted to pH 7.2 with 1 N NaOH), using an optical microscope and a scanning electron microscope. The color name used in this study was taken from Methuen Handbook of Colour[8]. Growth range of temperature was determined on yeast extract - malt extract agar. The preparation of cells and detection of the isomer of diaminopimelic acid was performed by procedure of

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BECKER *et al.*[9)].

Culture and Medium Conditions

A loopful of slant culture of *Streptomyces* sp. No. 9885 was inoculated into one 500-ml Erlenmeyer flask containing 160 ml of the first stage seed medium consisting of corn starch 1%, glucose 1%, peanuts powder 0.5%, yeast extract 0.5% and $CaCO_3$ 0.2%. The flask was incubated in a rotary shaker (220 rpm) at 30°C for 5 days. Further, 3.2 ml of the seed culture was transferred to fifteen 500-ml Erlenmeyer flasks containing 160 ml each of the second stage seed medium consisting of soluble starch 2%, glucose 1%, wheat germ 1%, yeast extract 0.5%, defatted soybean meal 0.5% and $CaCO_3$ 0.2%. The inoculated flasks were incubated in a rotary shaker (220 rpm) at 30°C for 2 days. The entire second seed culture was transferred to five 30-liter jar fermentors containing 100 liters of the production medium consisting of glucose 4%, dried yeast 1%, β-cyclodextrin 1%, $CaCO_3$ 0.2%, Adekanol LG-109 (Asahi Denka) 0.05% and Silicone of KM-70 (Shin-Etsu Kagaku) 0.05%. Cultivation was carried out at 30°C for 4 days at 200 rpm, one atom inner pressure and 20 liters/minute of an aeration volume.

Assay for FR182877 Production

The amount of FR182877 in the fermentation broth was determined by HPLC using YMC AM-303, S-5, A-120 column (4.6 mm inner diameter×250 mm length, YMC Co., Ltd.) at 210 nm with a mobile phase of 80% aqueous methanol and at a flow rate of 1 ml/minute. Retention time of FR182877 was 6.5 minutes.

Morphological Observation of BHK Cells

BHK cells were maintained and treated in suspension in DULBECCO's modified EAGLE's medium (DMEM) (Flow Laboratories, North Ryde, Australia) supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), and penicillin (50 units/ml) - streptomycin (50 μg/ml) (Flow Laboratories).

For the morphological observation, two-fold serial dilutions of FR182877 or other drugs were prepared in 96-well microplates containing the medium. BHK cells (5×10^3) in 100 μl of the medium were added and incubated for 48 hours in 5% CO_2 - 95% air atmosphere at 37°C. After the plates were fixed and treated with GIEMSA's stain solution, the morphological changes in BHK cells were examined under optical microscopy.

In Vitro Cytotoxicity Test

The cytotoxic activity of FR182877 *in vitro* was determined as follows. Concentration of the compound required for 50% inhibition of cell growth (IC_{50}; ng/ml) was determined by plotting the logarithms of the concentration vs. the growth rate (percentage of control) of the treated cells. Human mammary adenocarcinoma MCF-7 cells, human lung adenocarcinoma A549 cells or human colon adenocarcinoma HT-29 cells (4×10^4 cells/ml) were treated in suspension in 100 μl of DMEM supplemented with 10% FBS and the antibiotics (penicillin - streptomycin). Human T cell leukemia Jurkat (1×10^4 cells/ml), murine melanoma B16 cells or murine leukemia P388 cells (1×10^5 cells/ml) were treated in suspension in 100 μl of RPMI medium (Flow Laboratories) supplemented with 10% FBS and the antibiotics. The cells were grown in 5% CO_2 - 95% air atmosphere at 37°C. The cytotoxicity was colorimetrically determined at 550 nm (and 660 nm as a reference) according to MTT method described by MOSMANN[10)].

Evaluation of Antitumor Effects on Murine Ascitic Tumors

P388 leukemia cells (1×10^6) were i.p. implanted into BDF_1 mice. Five mice were used for each experimental group. FR182877 was solubilized in distilled water containing 20% polyethylene glycol #400. Twenty-four hours after the implantation of tumor cells, graded doses of FR182877 were administered to mice intraperitoneally. FR182877 was given once a day on Days 1, 4 and 7(Q3D).

Drug efficacy was assessed as a percentage of median survival time of the treated group (T) to that of the control group (C).

T/C (%)={Median survival time of (T)/Median survival time of (C)}×100

Evaluation of Antitumor Effects on Murine Solid Tumors

Fragments (2×2×2 mm) of Colon 38 cells were s.c. implanted into the left flank of BDF_1 mice. When the tumor weight in the mice had reached 100 to 300 mg, the mice were treated i.v. with FR182877 once a day for 4 days (QD, Days 1~4). The tumor weights were measured on Day 21. The tumor weight was calculated from the following formula: tumor weight (mg)$=1/2\times a\times b^2$ where a and b represent the length and the width of the tumor mass, respectively.

Drug efficacy was expressed as the percentage of mean tumor weight of the treated group (T) to that of the control group (C).

$$\text{Growth inhibition (\%)} = \left\{1-\frac{\text{change in mean tumor weight (T)}}{\text{change in mean tumor weight (C)}}\right\}\times 100$$

Antimicrobial Test

Antimicrobial activities of FR182877 were determined on Nutrient agar by a two-fold serial dilution method for bacteria and Sabouraud agar for fungi and yeast. The lowest concentration that inhibited growth of bacteria after 18 hours incubation at 37°C and of fungi and yeast after 48 hours incubation at 28°C is expressed in terms of μg/ml as the minimum inhibitory concentration (MIC).

Animals

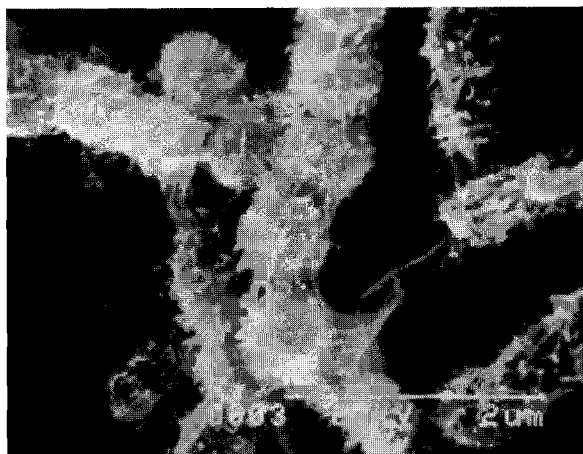
BDF_1 mice (female, 6 weeks old) were purchased from Charles River Japan Inc., Atsugi, Japan.

Analytical Measurement

Optical rotation was measured with a Jasco DIP-140 polarimeter using a 10-cm microcell. UV spectrum was obtained with a Hitachi 220A spectrometer. The mass spectrum was recorded with VG ZAB-SE mass spectrometer. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were acquired with a Bruker AM400wb spectrometer controlled with an ASPECT 3000 computer.

Fig. 1. Electron micrograph of strain No. 9885.

Bar represents 2 μm.



Results

Taxonomy of the Producing Strain

The substrate mycelium of strain No. 9885 developed well and branched irregularly. This strain produced aerial mycelium with spiral, loop and flexuous spore chains which comprised more than 10 spores per chain. Spores were oval ($0.5\sim0.6\times0.7\sim0.9\,\mu$m) in shape. The surfaces of them were hairy (Fig. 1). Sclerotic granules, sporangia and motile spores were not observed.

The cultural and physiological characteristics are shown in Tables 1 and 2, respectively. Aerial mass color was pale grey on yeast extract-starch-glucose agar. Reverse side color of growth was light brown, brown, and light orange. Melanoid pigments were not produced in trypton-yeast extract broth and pepton-yeast extract-iron agar. Soluble pigments were weakly produced in yeast extract-malt extract agar, oatmeal agar and inorganic salts-starch agar. These mycelial and soluble pigments were not pH sensitive. L,L-Diaminopimelic acid was detected in the whole-cell hydrolysates of this strain.

Based on the morphological and chemical characteristics described above, strain No. 9885 is considered to belong to the genus *Streptomyces*[11,12]. Therefore, this strain was designated as *Streptomyces* sp. No. 9885. The strain has been deposited in the Fermentation Research Institute, Agency of Industrial Science of Technology, Tsukuba, Japan, as *Streptomyces* sp. No. 9885 with the accession No. FERM BP-5007.

Table 1. Cultural characteristics of strain No. 9885.

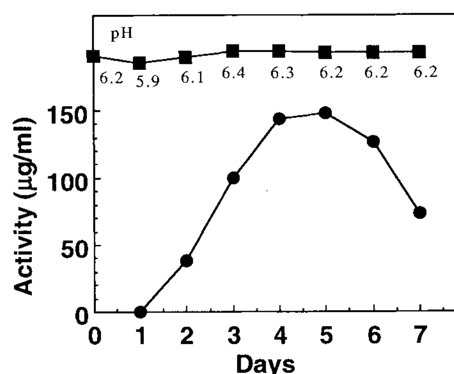
Medium	Growth	Aerial mycelium	Reverse side color	Soluble pigment
Yeast extract-malt extract agar	Moderate	None	Light brown (6D8)	Weak, reddish orange (7B7)
Oatmeal agar	Moderate	Very poor, white (1A1)	Brown (7E8)	Weak, brownish orange (7C7)
Inorganic salts-starch agar	Poor	None	Light orange (6A5)	Trace, reddish white (8A2)
Glycerol-asparagine agar	No growth			
Peptone-yeast extract-iron agar	Very poor	None	Colorless	None
Tyrosine agar	Poor	None	Colorless	None
Yeast extract-starch-glucose agar	Moderate	Poor, pale grey (1B1)	Brown (7B6)	None

Table 2. Physiological characteristics of strain No. 9885.

Condition	Characteristic
Temperature range for growth (℃)	13.5 ~ 36.5
Production of melanoid pigments	−
Production of soluble pigment	±
Starch hydrolysis	±
Carbon utilization	
D-Glucose	+
L-Arabinose	+
D-Xylose	+
Inositol	−
Sucrose	−
D-Fructose	±
D-Mannitol	−
L-Rhamnose	±
Raffinose	+
Glycerol	−

+ : positive, ± : weakly positive, − : negative

Fig. 2. Time course of FR182877 production (●) and pH change (■).



Fermentation of FR182877

Fig. 2 shows a typical time course of FR182877 production by *Streptomyces* sp. No. 9885 in a 30-liter jar fermentor, along with the pH of the medium. The production of FR182877 started at the second day and gradually increased for additional three days. The amount of FR182877 in the fermentation broth reached about 150 μg/ml at the fifth day.

Isolation

The fermentation broth (100 liters) was extracted with 100 liters of acetone and was filtered with the aid of diatomaceous earth. The filtrate was concentrated under reduced pressure and adjusted to pH 7.0 with 6 N-NaOH. The concentrate obtained was extracted twice with 100 liters of ethyl acetate. The extracts were combined for concentration under reduced pressure. The concentrate (43.2 g) was applied to a 600 ml of silica gel column, which prefilled with *n*-hexane. After washing with 2 liters each of *n*-hexane and *n*-hexane - ethyl acetate (1 : 1), the column was eluted with 2 liters of ethyl acetate. The eluted fraction was concentrated under reduced pressure, and the resulting oily material (17.2 g) was applied to a 200 ml of silica gel column, which prefilled with dichloromethane - methanol (25 : 1). After washing with 250 ml of dichloromethane - methanol (25 : 1), the column was eluted with the same solution. The active fractions were combined for concentration under reduced pressure. The active oily substance (6 g) was dissolved in 10 ml of methanol, and applied on a 350 ml of ODS gel (YMC ODS-AM 120-S50) column. The column was eluted with 60% aqueous methanol. After adding the equal volume of distilled water, the active fractions were applied to a Diaion HP-20 column and eluted with ethyl acetate. The extract was concentrated to dryness. The crude powder was dissolved in a small amount of ethyl acetate to obtain colorless needle-shaped crystals of FR182877 (238 mg).

Table 3. Physico-chemical properties of FR182877.

Appearance	colorless powder
Melting Point (℃)	164~167
$[\alpha]_D^{23}$	−3.5° (*c* 1.0, MeOH)
Molecular Fomula	$C_{24}H_{32}O_5$
Molecular weight	
HR-FABMS	
Calcd:	401.2328 $(M+H)^+$
Found:	401.2328 $(M+H)^+$
UV λ_{max}^{MeOH} nm(ε)	241 (1480), 285(2640)
Color Test	
Positive	$Ce(SO_4)_2$-H_2SO_4, I_2
Negative	Ninhydrin, Molish, $FeCl_3$, Ehrlich
Solubility	
Soluble	MeOH, EtOH, $(CH_3)_2CO$
	EtOAc, CH_2Cl_2
Slightly soluble	*n*-Hexane
Insoluble	H_2O
TLC (Rf value)	
System I[a]	0.36
System II[b]	0.30

[a] Plate: Silica gel 60 F_{254}(E. Merck Co.), CH_2Cl_2:MeOH=10:1
[b] Plate: RP-18 WF_{254}(E. Merck Co.), 80% aq. MeOH

Physico-chemical Properties

FR182877 is neutral in nature, and showed positive color reaction to iodine vapor and ceric sulfate though negative to ninhydrin. It is soluble in methanol, ethyl acetate and chloroform but insoluble in water. The physico-chemical properties were listed in Table 3. According to high-resolution FAB-MS spectrometry, the molecular formula is

Fig. 3. ^{1}H NMR spectrum of FR182877 (400 MHz, CD_3OD).

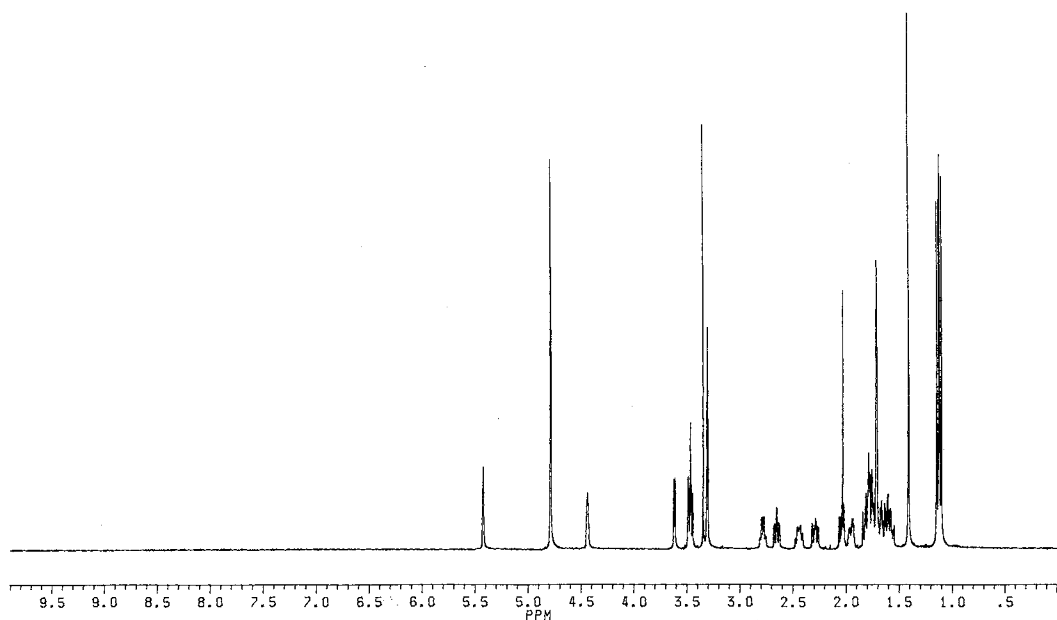
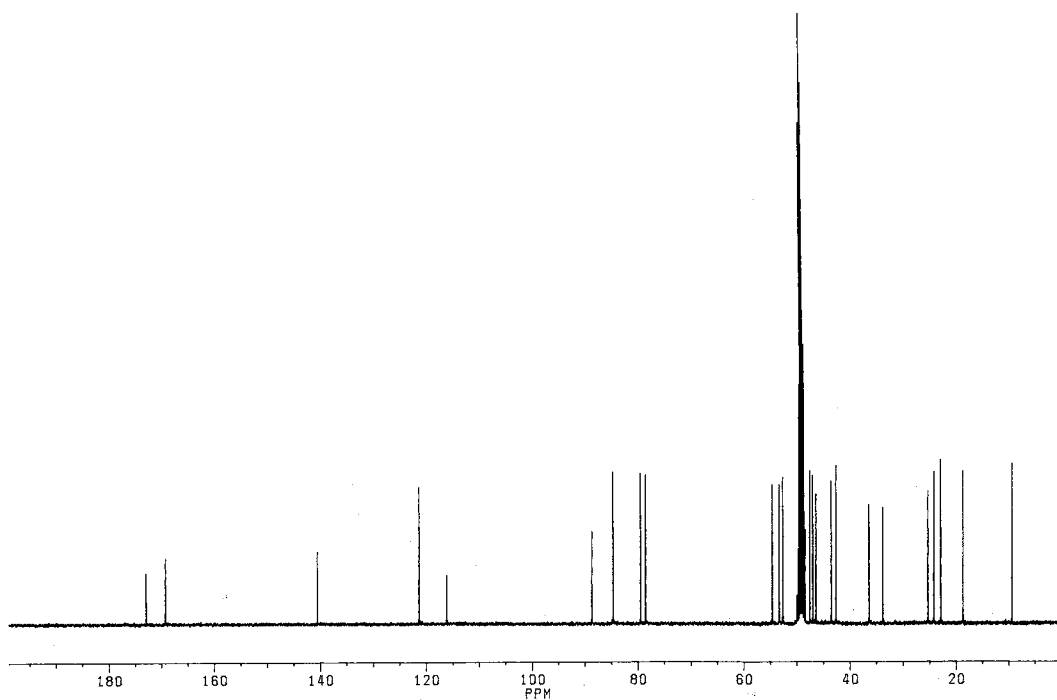
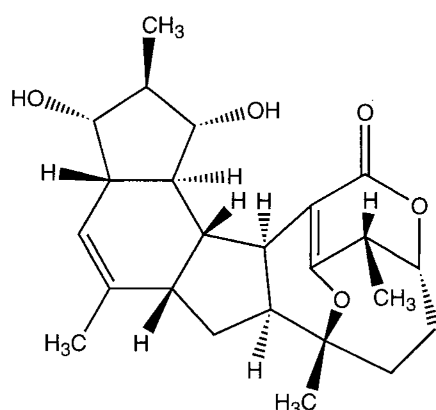


Fig. 4. ^{13}C NMR spectrum of FR182877 (100 MHz, CD_3OD).



$C_{24}H_{32}O_5$. In the ^{13}C NMR spectrum, all the ^{13}C signals were clearly observed. The ^{1}H and ^{13}C NMR spectra are reproduced in Figs. 3 and 4, respectively. The structure of FR182877 (see Fig. 5) was elucidated through the concerted application of a series of 2D NMR techniques and the relative stereochemistry was established by X-ray crystallographic analysis of its derivative. FR182877 has an unprecedented hexacyclic structure consisting of five-, six-, five-, six-, six-, and seven-membered rings. The highlight of its structure is a highly strained tetra-substituted double bond. The full account of the structure determination will be reported in this journal[13].

Fig. 5. Chemical structure of FR182877.



Biological Activities

Morphological Observation

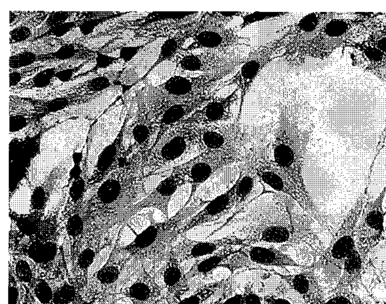
Morphological changes in BHK cells in the presence of FR182877 were examined. BHK cells were morphologically transformed to an amoebiform state at low concentration of FR182877. Furthermore, BHK cells formed multinuclear state when exposed to FR182877 at concentration in the vicinity of its IC_{50} (Fig. 6).

Antimitotic drugs in clinical use, vincristine (Shionogi & Co., Ltd.) and taxol (Bristol-Myers Squibb Co.) also caused the similar characteristic morphological changes in BHK cells (Fig. 6). However, DNA synthesis inhibitors, such as adriamycin (Kyowa Hakko Kogyo Co., Ltd.) and mitomycin (Kyowa Hakko Kogyo Co., Ltd.), did not caused these morphological changes (data not shown).

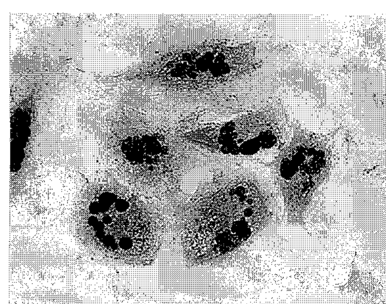
Cytotoxic Activity

The result was shown in Table 4. FR182877 was effective against the human tumor cell lines, though weaker against mouse bone marrow cells (IC_{50}: 600 ng/ml).

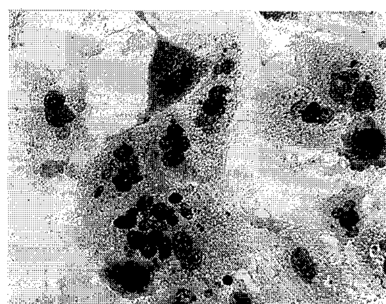
Fig. 6. Effects of FR182877 and other antimitotic drugs on morphological changes in BHK cells.



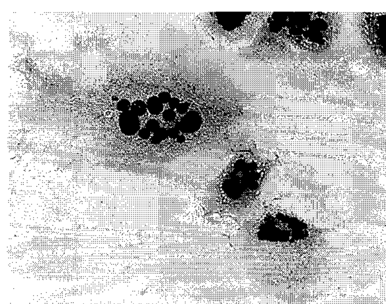
Control



FR182877



Vincristine



Taxol

Table 4. Cytotoxicity of FR182877 on various cell lines *in vitro*.

	IC_{50}(ng/ml)					
	MCF-7	A549	HT-29	Jurkat	P388	B16
FR182877	27	73	73	33	21	67

Antitumor Activities against Murine Tumor in Mice

The antitumor activities of FR182877 against murine ascitic tumor P388 and solid tumor Colon 38 were examined. As shown in Table 5, against P388 the FR182877 treatment prolonged the life span of the tumor bearing mice. The effective dose ranges were 1.6~6.3 mg/kg. Against Colon 38, FR182877 inhibited the growth of Colon 38 in the dose dependent manner and showed antitumor activity.

Antimicrobial Activity

FR182877 was devoid of antimicrobial activity when tested *vs.* the following microorganisms at 1000 μg/ml; *Escherichia coli* NIHJ JC-2, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* 209P, *Candida albicans* No. 7 and *Aspergillus fumigatus* FP1305.

Acute Toxicity

The acute toxicity of FR182877 was determined in mice by a single intraperitoneal injection of graded doses of FR182877 in the distilled water containing 20% polyethylene glycol #400. The LD_{50} was over 60 mg/kg.

Table 5. Antitumor effects of FR182877 on murine ascitic tumor (A) and solid tumor (B) *in vivo*.

A

P388 (i.p.-i.p.) Q3D[a] Days1, 4 and 7		
Dose (mg/kg)	FR182877	
	T/C(%)	Activity[b]
1.6	120	+
3.2	130	+
6.3	130	+
12.5	100	-
25.0	90	-
50.0	60	Tox[c]

a Q3D, every 3 day.
b Criteria : +, ≧120 and ++, ≧175
c Tox, a T/C value of <86% indicates toxicity

B

Colon 38 (s.c.-i.v.) QD[a] D1~4 Weight D21		
Dose (mg/kg)	FR182877	
	1-T/C(%)	Activity[b]
1.0	18	−
1.8	67	+
3.2		Tox[c]

a QD, every day.
b Tumor weights were measured on Day21. Criteria : +, >58 and ++, >90
c Tox, a survival rate of <65% on evaluation day indicates toxicity

Discussion

FR182877 was isolated from fermentation broth of *Streptomyces* sp. No. 9885 through a newly developed *in vitro* screening system for antimitotic substances. FR182877 caused characteristic morphological changes in BHK cells, which formed the multinuclear state when exposed at concentration in the vicinity of its IC_{50}. Taxol and vincristine also caused the similar morphological change. These morphological changes were thought to be caused by inhibition of mitosis in BHK cells. The details of the mechanism of action of FR182877 will be published in another paper[14]. Further, from the evidence of physicochemical data, it has a unique hexacyclic structure encompassing its highly strained double bond in chemical structure.

FR182877 prolonged the life span of mice bearing ascitic tumors and exhibited antitumor effect on murine solid

tumors. Thus, FR182877 is investigated further antitumor effects against murine and human solid tumors as a promising candidate compound.

Addedum in proof

FR182877 is identical to compound WS9885B[15,16].

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