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Migrastatin, a New Inhibitor of Tumor Cell Migration from *Streptomyces* sp. MK929-43F1

Taxonomy, Fermentation, Isolation and Biological Activities

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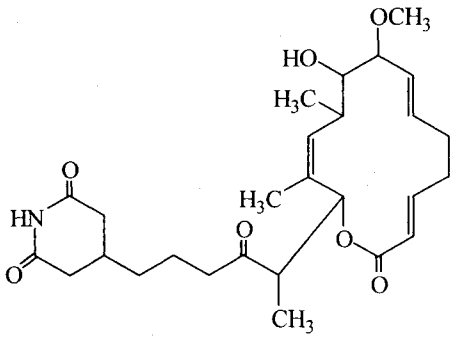
A new compound, migrastatin, was isolated from a cultured broth of *Streptomyces* sp. MK929-43F1, as an inhibitor of tumor cell migration. It was purified by column chromatographies on silica gel and Sephadex LH-20 and HPLC. Migrastatin has the molecular formula of $C_{27}H_{39}NO_7$ consisting of 14-membered macrolide and glutarimide moiety. It inhibited spontaneous migration of human esophageal cancer EC17 cells. Migration inhibitory activity of migrastatin was not dependent on cytotoxicity or inhibition of protein synthesis.

Despite significant advances in our understanding of the fundamental aspects of cancer, the development of metastatic lesions remains the predominant cause of death for most cancer patients. The development of metastasis requires a cell to complete a series of well-established steps[1]. In brief, a metastatic cell must escape from primary tumors, enter the circulation, invade into a tissue compartment and grow. Cell migration of tumor cells is essential for invasion of the extracellular matrix and for cell dissemination. Inhibition of the cell migration involved in the invasion process represents a potential therapeutic approach to the treatment of tumor metastasis. Therefore, we have screen the compounds which inhibit migration of tumor cells to develop a new anti-metastatic drug from microbial origin. Additionally, since signal transduction pathways involved in cell migration have not been well characterized[2], inhibitor of cell migration would be a useful tool for the elucidation of the mechanism of cell migration. In the course of screening for the cell migration inhibitor, we found that a strain of *Streptomyces* sp. MK929-43F1 produced a novel compound named migrastatin (Fig. 1). In this paper, the taxonomy of the producing strain, fermentation, isolation, and biological activities of migrastatin are described. The physicochemical properties and structure elucidation of migrastatin will be described in the following paper[3].

Fig. 1. Structure of migrastatin.



Dedicated to the memory of Sir EDWARD ABRAHAM.

Material and Methods

Microorganisms

Migrastatin producing organism, strain *Streptomyces* sp. MK929-43F1, was isolated form a soil sample collected in Atami-shi, Shizuoka Prefecture, Japan.

Taxonomic Studies

Cultural and physiological characteristics were determined by the method of SHIRLING and GOTTLIEB[4] and by the methods of WAKSMAN[5]. Carbohydrate utilization was investigated by using the procedure of PRIDHAM and GOTTLIEB[6]. Morphological characteristics were observed with a scanning electron microscope (Hitachi S-570). 2,6-Diaminopimelic acid in the cell wall was analyzed from the hydrolysate of the culture growth according to the method of STANECK and ROBERTS[7]. Menaquinone was analyzed by the methods of TAMAOKA *et al.*[8].

Cell Culture

Human esophageal cancer EC17 cells (provided by Dr. I. B. WEINSTEIN, Columbia University, NY)[9] were grown in RPMI 1640 medium containing 5% fetal bovine serum (FBS) and mouse melanoma B16 cells were grown in DMEM medium containing 10% fetal bovine serum (FBS), and both cells were maintained in a 37°C incubator with 5% CO_2.

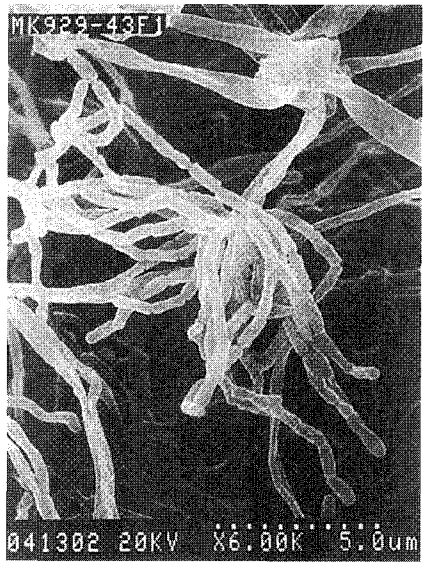
Wound Healing Assay

EC17 cells (6×10^4 cells/48 well plate) were plated in dishes, and after 24 hours of incubation, the confluent monolayer of cells was scratched with a white tip to create a cell-free zone in each well, about 1 mm in width. The medium was aspirated and replaces with medium containing 1% FBS in the presence or absence of screening sample. After 24 hours, cells were observed under microscope and were taken photographs[10].

Chemotaxicell Chamber Assay

Chemotactic migration of cells was assayed in a chemotaxicell chamber by previously reported with some modification[11]. Briefly, EC17 cells (1.8×10^5 cells) or B16 (1.8×10^5 cells) suspended in medium containing 0.1% FBS was added to the upper compartment of the chamber, and incubated with conditioned medium of EC17 cells or NIH3T3 cells in the lower compartment for 9~18 hours at 37°C in a 5% CO_2 atmosphere. The filter was fixed with MeOH and stained with hematoxylin. The cells on the upper surface of the filter were removed by wiping with cotton swabs. The cells that migrated through the filter to the various areas of the lower surface were counted manually under a microscope at a magnification of ×200.

Fig. 2. Scanning electron micrograph of *Streptomyces* sp. MK929-43F1.



Macromolecular Synthesis

EC17 cells were plated in dishes 24 hours before use and labeled with 1 μCi/ml of [^{3}H]thymidine, [^{3}H]uridine or [^{3}H] leucine in the presence of various concentrations of migrastatin. After 60 minutes, the medium was removed and the cells were treated with 0.5 ml of cold 10% trichloroacetic acid. The acid-insoluble fraction was dissolved in 0.5 N NaOH and then counted with a scintillation counter.

Results

Taxonomic Studies

The strain MK929-43F1 produced branched vegetative mycelia. This strain formed relatively long aerial hyphae, which formed verticillate sporophore. Mature spore chains consisted of 3 to 10 or more spores. The spore was 0.4~0.7×1.1~1.4 μm in size with smooth surface. Sporangia, motile spores or synnemata were not observed (Fig. 2).

The cultural characteristics of the strain MK929-43F1 on various agar media are summarized in Table 1. The vegetative growth color was pale yellow to pale yellowish brown on various media tested. The aerial mycelium was white to yellowish gray.

Table 1. Cultural characteristics of strain MK929-43F1.

	Growth	Aerial mycelium	Soluble pigmemt
Sucrose-nitrate agar	Colorless	Thin, white ~ cottony, yellowish gray [1 cb, Parchment]	None
Yeast extract-malt extract agar (ISP No.2)	Pale yellowish brown [2 ie, Lt Mustard Tan]	Thin, white ~ yellowish gray [1 cb, Parchment]	None
Oatmeal agar (ISP No.3)	Pale yellow [1 ca, Pale Yellow] ~ pale yellowish brown [2 gc, Bamboo]	Scant, white	None
Inorganic salts-starch agar (ISP No.4)	Pale yellow [1 ca, Pale Yellow]	Cottony, yellowish gray [1 1/2 ca, Cream]	None
Glycerol-asparagine agar (ISP No.5)	Pale yellow [1 ca, Pale Yellow ~ 1 1/2 ea, Lt Yellow]	Yellowish gray [1 1/2 ca, Cream]	None
Tyrosine agar (ISP No.7)	Pale yellow [1 ca, Pale Yellow] ~ grayish yellow brown [2 ig, Slate Tan]	Thin, yellowish gray [1 1/2 ca, Cream]	Faint, brownish

Observation after incubation at 27℃ for 21 days.

Color names and numbers from Color Harmony Manual, Container Corporation of America.

Table 2. Physiological characteristics of strain MK929-43F1.

Temperature range for growth(℃)	10 ~37
Optimum temperature(℃)	30
Formation of melanoid pigment	
ISP No.1	Positive
ISP No.6	Positive
ISP No.7	Negative
Hydrolysis of starch	Positive
Reduction of nitrate	Negative
Utilization of *	
D-Glucose	+
L-Arabinose	-
D-Xylose	-
D-Fructose	(+)
Sucrose	-
Inositol	+
Rhamnose	-
Raffinose	-
D-Mannitol	-

* : +, Utilization; (+), Probably utilization; -, No utilization.

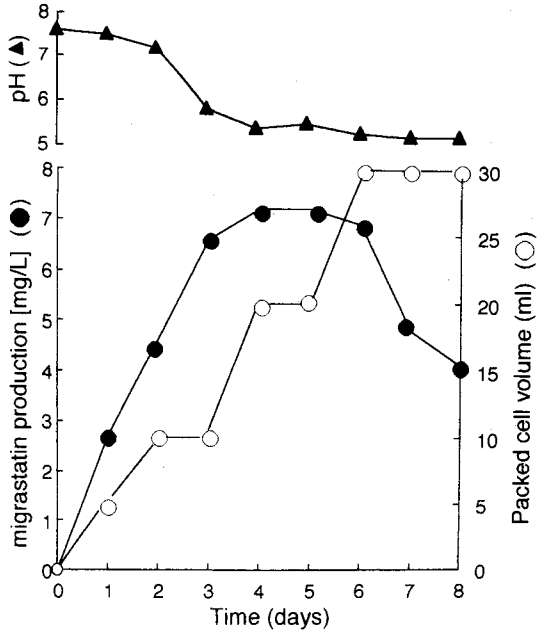
The physiological characteristics and carbohydrate utilization of strain MK929-43F1 were shown in Table 2. Optimum temperature for growth was 30°C. Formation of melanoid pigment was positive on ISP No. 1 medium and ISP No. 6 medium. Hydrolysis of starch was positive.

Analysis of whole-cell hydrolysate of the strain showed the presence of LL-diaminopimelic acid. The predominant menaquinone was MK-9(H_6). Based on these characteristics, strain MK929-43F1 was considered to belong to the genus *Streptomyces*. This strain was deposited in National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, under the name of *Streptomyces* sp. MK939-43F1 and the accession No. of FERM P-17574. *Streptomyces* sp. MK929-43F1 is similar to *Streptomyces caespitosus*, as judged from whorl-formation, yellow pigment of vegetative mycelium, carbohydrate utilization and mitomycin C production.

Fermentation

Streptomyces sp. MK929-43F1 was inoculated in a 500 ml Erlenmeyer flask containing 110 ml of a seed medium composed of glycerol 2%, dextrin 2%, Soytone Peptone (Difco) 1%, yeast extract 0.3%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.2 %, (adjusted to pH 7.4 before sterilization). The inoculated medium was incubated at 27°C for 48 hours on rotary shaker (220 rpm). Two ml aliquots of this seed culture was inoculated into 110 ml of same medium in a 500 ml Erlenmeyer flask, and incubated at 27°C for 5 days on a rotary shaker (220 rpm). A typical time course for

Fig. 3. A typical time course of migrastatin production by *Streptomyces* sp. MK929-43F1.



production of migrastatin was shown in Fig. 3. The production of migrastatin was measured by HPLC under the following conditions: column, Capcell pak (4.6×150 mm); solvent, 65% aq acetonitrile; detection, UV at 220 nm. The production of migrastatin began at day 1 after inoculation, and reached a maximum at day 5.

Isolation

The cultured broth (6 liters) was centrifuged to separate a supernatant and a mycelial cake. The supernatant was adjusted to pH 8.0 with NaOH and was extracted with EtOAc (6 liters). The extract was dried over anhydrous Na_2SO_4 and evaporated to dryness to give yellow oil (1.02 g). The yellow oil was loaded on a silica gel column, and active fraction was eluted with $CHCl_3$-MeOH (100 : 1). The eluate was concentrated *in vacuo* to yield a yellow powder (629.0 mg). It was applied on Sephadex LH-20 column using MeOH as the eluent. Active fraction was concentrated *in vacuo* to give a yellow powder (113.7 mg). The yellow powder, thus obtained, was further purified on HPLC (Capcell Pak C_{18} column, 20×250 mm) developing with 65% aq acetonitrile. Active fractions were combined and concentrated *in vacuo* to yield a pure colorless powder of migrastatin (20.0 mg).

Biological Activities

Effect of Migrastatin on Cell Migration of Cancer Cells

To examine the effect of migrastatin on EC17 cell migration, we performed two distinct migration assays; wound healing assay and chemotaxicell chamber assay. In the *in vitro* wound healing assay, EC17 cells were plated densely in tissue culture and after 24 hours a standardized scratch was made through the confluent monolayer. The cells from the cut edge of the scratch were then allowed to migrate for 24 hours. EC17 cells migrated inwardly and covered a great area of scratch, as shown in Fig. 4. One μg/ml of migrastatin significantly inhibited the migration of EC17 cells, and 3.0 μg/ml of migrastatin completely inhibited cell migration as judged from the residual area between the inwardly migrating EC17 cells from the edges of the scratch. Migration inhibitory activity of migrastatin was further confirmed by chemotaxicell chamber assay. In this assay, EC17 cells in the top chamber migrated and penetrated the filters to enter the lower chamber supplemented with the conditioned medium of EC17 cells, as determined by counting the number of cells attached to the lower side of the filter after 9 hours incubation. Migrastatin inhibited migration with an IC_{50} of 1.0 μg/ml, as shown in Fig. 5. Next we examined the effect of migrastatin on cell migration of other type of cell by using chemotaxicell chamber assay. B16 melanoma cells in top chamber migrated and penetrated into the filter to enter the lower chamber which was supplemented with the conditioned medium of NIH3T3 cells after 24 hours incubation. Migrastatin also inhibited the migration of B16 melanoma cells with an IC_{50} of about 3.0 μg/ml (Fig. 5).

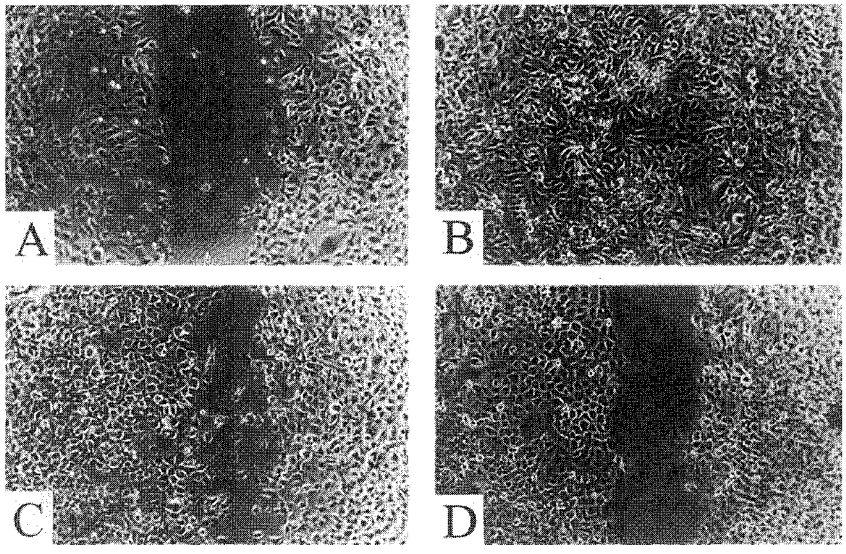
Effect of Migrastatin on Cell Viability of Tumor Cells

EC17 or B16 cells were incubated with various concentrations of migrastatin under the condition where migrastatin inhibited cell migration of each cell. Migrastatin did not show significant effect on viability of EC17 or B16 cells, as estimated by trypan blue dye exclusion assay (Fig. 5). Further incubation (48 hours) of cells with migrastatin (30 μg/ml) caused only weak reduction of cell viability of EC17 cells and B16 melanoma cells, as well as human carcinoma A431 cells, human colon carcinoma HT29 cells, and rat fibroblast 3Y1 cells.

Effect of Migrastatin on Macromolecular Synthesis

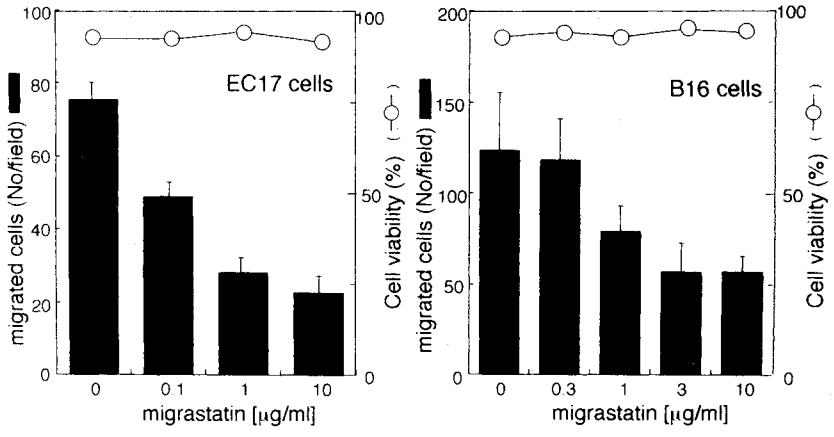
Migrastatin was tested for its inhibitory activities on macromolecular synthesis (Fig. 6). Migrastatin did not inhibit DNA synthesis up to 30 μg/ml. At 30 μg/ml migrastatin caused slight inhibition (25%) of RNA

Fig. 4. Effect of migrastatin on EC17 cell migration by wound healing assay.



Wound was introduced in confluent culture of EC17 cells, as described in "Materials and Methods" (A). The cells were incubated without (B) or with 1.0 (C), or 3.0 (D) μg/ml of migrastatin, respectively. After 24 hours, the cells were photographed under phase-contrast microscopy.

Fig. 5. Effect of migrastatin on migration of tumor cells by chemotaxicell chamber assay.



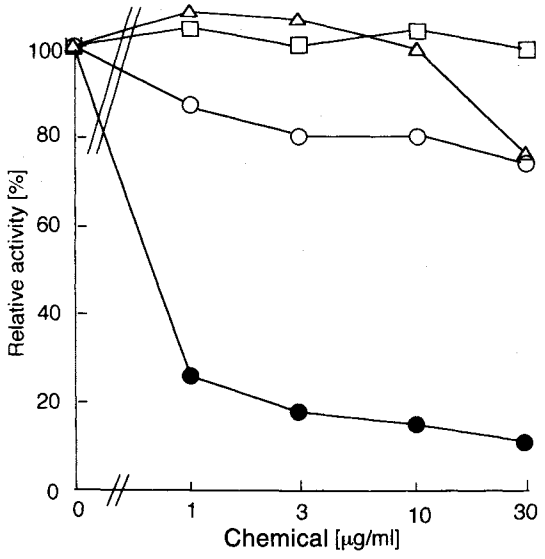
EC17 cells (left) and B16 cells (right) were incubated with various concentrations of migrastatin in top chamber for 9 hours (EC17 cells) or 18 hours (B16 cells). After then, the cells that migrated through the filter to lower surface were counted (bar). Cell viability was assessed by trypan blue dye exclusion assay. Values are means±SD for triplicate samples.

synthesis. Protein synthesis was also slightly inhibited by migrastatin, however, migrastatin was significantly less active than cycloheximide.

Discussion

Migrastatin, a novel glitarimide antibiotic having a unique 14-membered lactone ring, was isolated from the

Fig. 6. Effect of migrastatin on macromolecular synthesis of EC17 cells.



Cells were labeled with each precursor in the presence of various concentrations of migrastatin or cycloheximide for 60 minutes. After then, the radioactivity of TCA-insoluble fraction was counted. DNA (migrastatin), □; RNA (migrastatin), △; protein (migrastatin), ○; protein (cycloheximide), ●. Values are means of two independent determinations.

fermentation broth of *Streptomyces* sp. MK929-43F1 as an inhibitor of cell migration of tumor cells. Migrastatin inhibited cell migration of human esophageal cancer EC17 cells and mouse melanoma B16 cells as estimated by both wound healing assay and chemotaxicell chamber assay. These inhibitory effects of migrastatin should not be due to the toxic effect of the drug because more than 90% of cells being viable under same conditions. Another 14-membered ring macrolides, such as clarithromycin and erythromycin, did not inhibit cell migration of both EC17 and B16 cells up to 100 μg/ml (data not shown). Because migrastatin is related to the glutarimide antibiotic, we next examined the inhibitory activity of migrastatin on protein synthesis. When compared with cycloheximide, a glutarimide inhibitor of eukaryotic protein synthesis, migrastatin showed insignificant inhibition of protein synthesis as well as DNA and RNA synthesis up to 30 μg/ml. These results indicated that inhibition of migration by migrastatin was not due to the inhibition of protein synthesis. Indeed, cycloheximide did not inhibit cell migration of EC17 cells. Among the glutarimide class antibiotics, lactimidomycin[13] having a 12-membered lactone ring, has reported to exhibit both strong cytotoxicity against various tumor cells and antifungal activity, but no antibacterial activity. Migrastatin also did not show antibacterial activity, however, migrastatin is differentiated from lactimidomycin by its very weak cytotoxicity and its no antifungal activity (data not shown). Dorrigocin A is also glutarimide antibiotic having an acyclic unsaturated ketone side chain showing a similar structure to a hydrolysate of 14-membered lactone ring of migrastatin. It is reported to be an inhibitor of carboxyl methylation of Ras-related proteins, resulting in morphological reversion of *ras*-transformed cells[14,15]. We don't know at present whether migrastatin has the similar activity, however, migrastatin did not induce phenotypic reversion of Ha-*ras*-transformed 3Y1 and NIH3T3 cells. Thus, migrastatin is a novel glutarimide antibiotic having 14-membered macrolide, and shows a unique biological properties. Migrastatin would provide a valuable tool for further studies toward understanding the mechanism of tumor cell migration. In addition, migrastatin is potentially a useful compound in treating metastasis. Detailed studies on biological activities *in vivo* and *in vitro* are in progress.

Acknowledgment

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