A NEW ANTITUMOR ANTIBIOTIC, STUBOMYCIN

IWAO UMEZAWA, HIDEO TAKESHIMA, KANKI KOMIYAMA, YOSHIE KOH, HIROSHI YAMAMOTO and MASAE KAWAGUCHI

The Kitasato Institute, Shirokane, Minato-ku, Tokyo, Japan

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A new antibiotic, stubomycin, was isolated from the culture broth and mycelia of *Streptomyces* strain No. KG-2245. Stubomycin was prepared as colorless plates and has the empirical formula $C_{29}H_{35}NO_5$. The antibiotic possesses growth inhibitory activity against Gram-positive bacteria and transplantable murine tumors, such as Ehrlich carcinoma, and leukemia P388. The antibiotic also shows direct cytotoxic activity against HeLa cells *in vitro*.

In our search for new antitumor antibiotics, we isolated a new substance from the culture filtrate and mycelia of *Streptomyces* strain No. KG-2245 isolated from a soil sample. This paper describes a taxonomic study of the producing organism, and gives physico-chemical and biological properties of a new antibiotic, stubomycin.

Materials and Methods

Taxonomic Studies

For taxonomic studies, most cultures were grown in accordance with methods adopted by the International Streptomyces Project.[1] For experiments on cultural properties, all cultures were incubated at 27°C and were observed for 15~20 days. The color recorded for mature cultures is described according to the "Color Harmony Manual"[2]. Physiological properties including the utilization of carbon sources were examined by the method of PRIDHAM and GOTTLIEB.[3]

Diaminopimelic acid in the cell wall was analysed by the method of BECKER *et al.*[4]

Fermentation and Isolation of Stubomycin

Stock cultures of the producing organism were inoculated into a 500-ml Sakaguchi flask containing 125 ml of CZAPEK solution containing 2% glucose, 1% soy bean meal, 0.3% peptone, 0.3% NaCl, and 0.3% $CaCO_3$. The flask was incubated at 27°C for 96 hours on a reciprocal shaker, and 2 ml of the resulting culture was transferred into 500-ml Sakaguchi flasks each containing the same medium described above. The flasks were incubated at 27°C for 48 hours on a reciprocal shaker. To 20 liters of fermentation medium containing 2% glucose, 1% soy bean meal, 0.3% dry yeast, 0.3% NaCl, and 0.3% $CaCO_3$ was added 400 ml of the resulting inoculum and the culture was incubated at 27°C in a 30-liter jar fermentor for 70 hours, aerated at 13 liters/minute, and agitated at 300 rpm. The broth filtrate (16 liters) was extracted twice with 3 liters of ethyl acetate. After the evaporation of the solvent, the extract was treated with *n*-hexane, and a crude powder was obtained. The resulting powder was dissolved in MeOH, and was subjected to Sephadex LH-20 column chromatography. The active fraction was concentrated *in vacuo* and the residue was rechromatographed on Sephadex LH-20. After evaporating the solvent layer, the crude powder was further purified on a column with silica gel treated with 2 N H_2SO_4 before use. Stubomycin was eluted with dichloromethane. After the solvent was concentrated *in vacuo* the crystals of the antibiotic were obtained. Recrystallization from MeOH gave colorless plates.

Besides this, stubomycin was also isolated from mycelia as shown in Fig. 1.

Antimicrobial Activity

The antimicrobial spectrum of stubomycin was determined by the agar dilution method using

nutrient agar for bacteria and potato agar for fungi. The minimum inhibitory concentration was observed after 48 hours incubation.

Antitumor Activity

For the determination of antitumor activity of the antibiotic, male *dd*Y mice and CDF_1 mice weighing 20~24 g were obtained from Shizuoka Agricultural Cooperation. Ehrlich ascites carcinoma and P388 lymphocytic leukemia cells have been maintained by intraperitoneal (i.p.) injection in *dd*Y mice or DBA mice, respectively. *dd*Y Mice or CDF_1 mice were inoculated i.p. with Ehrlich ascites carcinoma cells (2.5×10^6) or P388 lymphocytic leukemia cells (1×10^5), respectively. Groups of 7 mice for each dosage regimen were given i.p. injections of 0.1 ml/10 g body weight of the antibiotic solution from days 1 through 9, days 1, 5, and 9 or day 1 only, then survival period was observed.

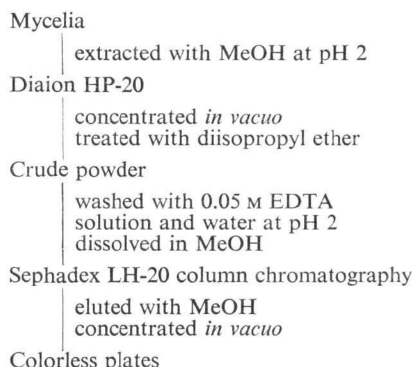


Fig. 1. Purification method of stubomycin from mycelia.

Effect of Stubomycin on HeLa Cells

HeLa cells have been maintained in monolayers in EAGLE's minimum essential medium (MEM) supplemented with 10% bovine serum and antibiotics (100 unit/ml of penicillin and 100 μg/ml of streptomycin) at 37°C. In order to determine the cytotoxicity of stubomycin on mammalian cells, HeLa cells (5×10^4) in 2 ml of medium were placed into 30-mm Petri dish and incubated for 48 hours at 37°C in 5% CO_2 - 95% air atmosphere. Each culture dish was refed with a fresh medium containing a different concentration of stubomycin. Then HeLa cells were exposed to the antibiotic for 24, 48 or 72 hours. After exposure, each monolayer culture was trypsinized to form a single cell suspension, and cells were counted by a hemocytometer.

Determination of the Synthesis of Cellular Macromolecules

[^{3}H]-Thymidine (^{3}H-TdR, 27 Ci/mmol), [^{3}H]-uridine (^{3}H-UR, 30 Ci/mmol), and [^{3}H]-leucine (^{3}H-Leu, 46 Ci/mmol) were obtained from the Radiochemical Center, Amersham, UK.

HeLa cells (3×10^4) were plated into 30-mm Petri dish, each inserted with 18 mm cover glass with 2 ml of MEM and allowed to reach exponential growth (48 hours). Triplicate specimens were treated with ^{3}H-TdR (0.5 μCi/ml), ^{3}H-UR (0.5 μCi/ml) or ^{3}H-Leu (1 μCi/ml) for 30 minutes at 4 hours after the addition of stubomycin. Then the cells were rinsed 3 times with ice-cold 5% trichloroacetic acid. The radioactivity of acid-precipitable material was determined by the Aloca gas-flow counter.

Results

Taxonomic Studies

Microscopic examination revealed that the aerial mycelia were mostly rectiflexibiles with hooks on oat meal agar, but some retinaculiaperti or spirals were observed in mycelia grown on WAKSMAN's agar (Plate 1). Viewed under the transmission microscope, spores were cylindrical, and the sizes were 0.47~0.67×1.2~1.6 μ. As shown in Plate 2, the spore surface was smooth with minor irregularities (neither smooth nor typical warty or spiny).

Table 1 shows the cultural properties of strain No. KG-2245. Growth on chemically defined media as poor compared with that on natural nutrient media. The physiological properties and utilization of carbon sources of strain No. KG-2245 are shown in Tables 2 and 3. The analysis of cell wall composition found LL-diaminopimelic acid and classified as Type I. Microscopic studies and cell wall type indicated that strain No. KG-2245 belongs to the genus *Streptomyces* and designated as *Streptomyces* sp. KG-2245. Further studies are in process.

Plate 1. Aerial mycelium of strain No. KG-2245 (×2,000).

Plate 2. Spore surface of strain No. KG-2245 (×16,000).

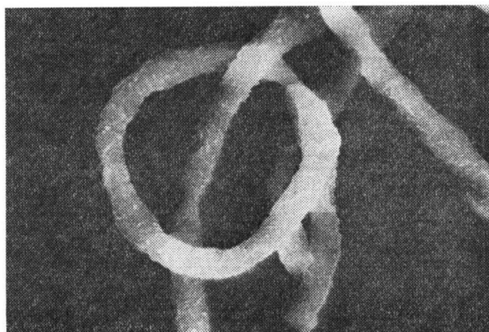


Table 1. Cultural properties of strain No. KG-2245.

Medium	Growth	Aerial mycelium	Riverse	Soluble pigment
Sucrose-nitrate agar	Poor	Ashes (5fe)	Silver gray (3fe)	Bamboo (2fb)
Glucose-nitrate agar	Moderate	Pussywillow gray (5dc)	Dusty orange (4lc)	Pastel orange (4ic)
Glycerol-calcium malate agar	Moderate	Maple (4le)	Light coral rose (6ga)	—
Glucose-asparagine agar	Good	Marigold (3na)	Yellow maple (3ng)	Amber (31c)
Glycerol-asparagine agar	Good	Colonial yellow (2ga)	Amber (3pe)	Maize (2hb)
Inorganic salts-starch agar	Very poor	—	Orchid tint (10ba)	—
Tyrosine agar	Good	Oatmeal (2ec)	Yellow maple (3ng)	Maize (2hb)
Yeast extract-malt extract agar	Moderate	Rose beige (4gc)	Tile red (5ne)	Peach pink (5ea)
Oat meal agar	Moderate	Natural (3dc)	Sand (2ec)	Light beige (3ec)
Peptone-yeast extract-iron agar	Moderate	Ivory (2db)	Maize (2hb)	±
Glucose peptone agar	Moderate	Orchid tint (10ba)	Bamboo (2fb)	Melon yellow (3ga)
Nutrient agar	Moderate	Rose beige (4gc)	Orange (4la)	Pastel orange (4ic)

Table 2. Physiological characteristics.

Nitrate reduction	+
Liquefaction of gelatin	—
Coagulation of milk	—
Cellulolytic activity	±
Melanin formation	—

Table 3. Utilization of carbon sources.

Responses	Carbon source
Positive	D-glucose, *i*-inositol
Negative	L-arabinose, sucrose, raffinose, D-mannitol, D-fructose, cellulose
Doubtful	D-xylose, rhamnose

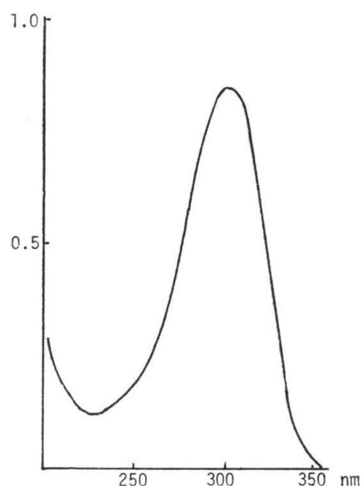
Physico-chemical Properties of Stubomycin

The physico-chemical properties of the antibiotic are listed in Table 4; UV, IR and NMR spectra are shown in Figs. 2, 3 and 4. As shown in Fig. 2, UV maximum in MeOH was 300 nm and this peak did not shift at alkaline or acidic conditions. Stubomycin is readily soluble in dimethylsulfoxide, pyridine, and dimethylformamide, slightly soluble in MeOH, and insoluble in water or organic solvents such as chloroform or ether. Stubomycin gives positive RYDON-SMITH, DRAGENDORFF, ferric chloride, and 2,4-dinitrophenylhydrazine, but negative ninhydrin, EHRLICH, anisaldehyde-H_2SO_4 reactions.

Table 4. Physico-chemical properties of stubomycin.

Molecular formula	$C_{29}H_{35}NO_5$
Analysis Found.	C 72.33, H 7.35, N 2.83 %
Calcd.	C 72.58, H 7.47, N 2.80 %
Melting point	243~245°C (dec.).
$[\alpha]_D^{20}$ (c 0.5, DMSO)	+246°
IR (KBr) cm^{-1}	1,640, 1,600~1,610, 1,120, 1,005
UV nm ($E_{1cm}^{1\%}$) MeOH	300 (930)

Fig. 2. UV spectrum of stubomycin (MeOH).



The behavior on thin-layer chromatography was Rf 0.5 (Merck silica gel 60F-254, solvent: $CHCl_3$ - MeOH - AcOH, 91.5: 7: 1.5).

The molecular weight was 477 by mass spectrum, and no sulfur and chlorine were contained. From molecular weight and elemental analysis, the molecular formula was determined as $C_{29}H_{35}NO_5$. Though the data was not shown, this formula was confirmed by ^{13}C NMR of stubomycin diacetate. Structural studies in progress suggest that this antibiotic contains a polyene amide group (–NH–CO–(C=C)$_n$–) and a phenyl group.

Biological Properties of Stubomycin

The antibiotic is active against Gram-positive bacteria and weakly active against certain fungi, but inactive against a few Gram-negative bacteria (Table 5). The LD_{50}s of the antibiotic on *dd*Y mice were about 500 mg/kg and over 1,000 mg/kg upon injection i.p. and p.o., respectively.

Antitumor activity of stubomycin on Ehrlich ascites carcinoma and P388 leukemia was shown in Table 6. The antibiotic produced a prolongation of mean survival time in all treatment schedules, and at the appropriate dose, daily and a single injection was almost equally effective. Stubomycin

Fig. 3. IR spectrum of stubomycin (KBr).

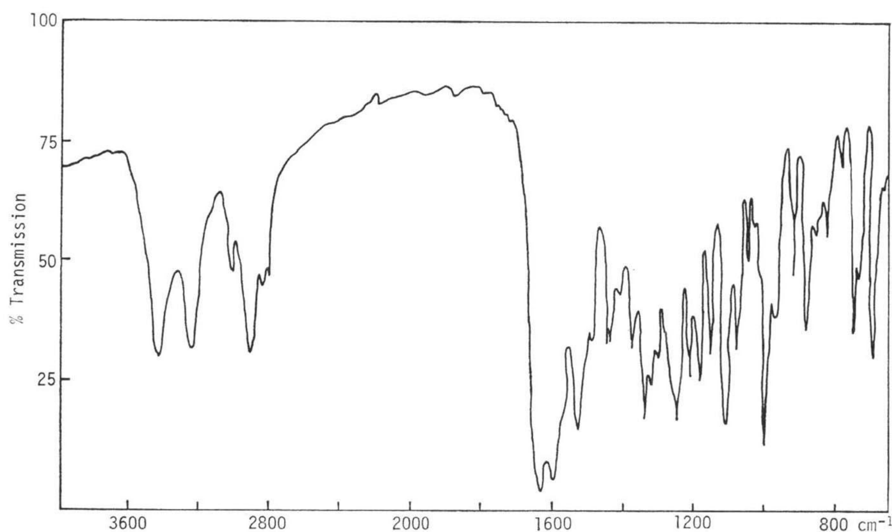


Fig. 4. NMR spectrum of stubomycin (100 MHz, DMSO-d_6).

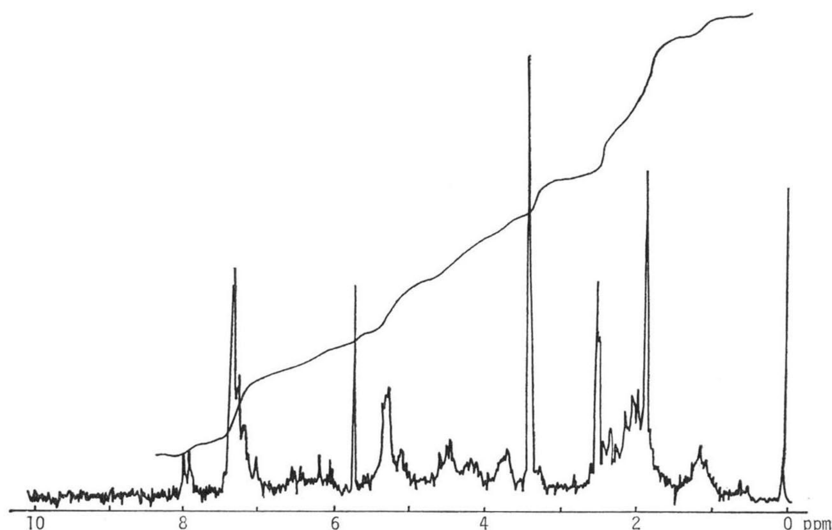


Table 5. Antimicrobial activity of stubomycin.

Test organism	MIC (μg/ml)	Test organism	MIC (μg/ml)
Bacillus subtilis PCI 219	0.4	*Candida albicans*	>100
Staphylococcus aureus FDA-209P	3.1	*Saccharomyces sake*	>200
Mycobacterium smegmatis	3.1	*Aspergillus niger*	>100
Sarcina lutea ATCC 1001	0.4	*Trichophyton interdigitale*	6.3
Escherichia coli NIHJ	>200	*Piricularia oryzae*	3.1
Pseudomonas aeruginosa	>200	*Alternaria kikuchiana*	6.3
Xanthomonas oryzae	>100	*Microsporum gypseum*	12.5

Table 6. Antitumor activities of stubomycin on Ehrlich ascites carcinoma and leukemia P388.

Treatment schedule	Dose (mg/kg/day)	Total dose (mg/kg)	MSD		ILS	
			Ehrlich	P 388	Ehrlich	P 388
Untreated	—	—	19	12	—	—
Day 1	75	75	28 (1)*	17	47	42
	150	150	51	17	168	42
	300	300	21	17	11	42
Days 1, 5, 9	25	75	33 (1)	16	74	33
	50	150	16	15	−16	25
Days 1∼9	8.8	75	21	15	11	25
	16.7	150	50 (1)	11	163	−8
	33.3	300	33 (1)	9	74	−25

MSD: Median survival day.
ILS: Increased life span, expressed as percent increase over the untreated control mice.
* Number of cured mice.

Fig. 5. Cytocidal activity of stubomycin on HeLa cells.

Numbers in figure indicate concentration (μg/ml) of the antibiotic.

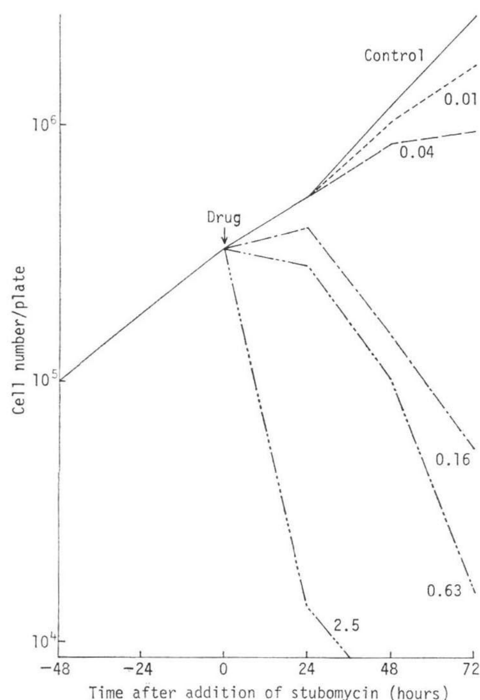
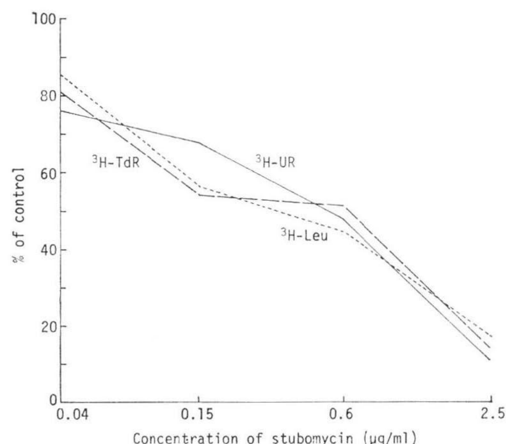


Fig. 6. Effect of stubomycin on the synthesis of macromolecules in HeLa cells.



also showed antitumor activity against P388 leukemia.

The effect of adding stubomycin to asynchronous, exponentially growing cultures of HeLa cells was determined. Fig. 5 shows that a concentration of 0.16 μg/ml was effective in completely preventing the cell division, while 0.04 μg/ml was only partially effective.

In order to determine the effect of stubomycin on the synthesis of cellular macromolecules, HeLa cells were incubated with ^{3}H-TdR, ^{3}H-UR, or ^{3}H-Leu for 30 minutes after the cells were preincubated for 4 hours in the presence of the antibiotic, and the incorporation ratio was compared with that of non-treated cells. As shown in Fig. 6, the incorporation of the precursors into cellular macromolecules was strongly inhibited at a concentration of 2.5 μg/ml of the antibiotic, and incorporation ratio was almost the same among these precursors at various concentrations of the antibiotic.

Discussion

According to the physico-chemical properties described above and the data of structural studies on stubomycin, the antibiotic contains a polyene amide group. Among the known antibiotics, stubomycin is similar to viridenomycin.[5,6] Viridenomycin shows the maximum at 310 nm in UV absorption, and its m.p. and formula are 168~171°C and $C_{34}H_{37}NO_7$, respectively. Stubomycin showed m.p. 243~245°C and $C_{29}H_{35}NO_5$. Stubomycin was also compared with other known antibiotics that have ultraviolet absorptions around 300 nm and the property of being insoluble in water. Several antibiotics such as inomycin (300 nm)[7], eumycetin (302 nm)[8], pyridomycin (303 nm)[9] and seligocidin (304 nm)[10] were examined. However, none of their properties as identical with any of those of stubomycin.

From these data stubomycin is believed to be a new antibiotic.

Acknowledgement

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