RESORTHIOMYCIN, A NOVEL ANTITUMOR ANTIBIOTIC

I. TAXONOMY, ISOLATION AND BIOLOGICAL ACTIVITY

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Resorthiomycin, a novel antitumor antibiotic, was isolated from the fermentation broth of a strain of *Streptomyces collinus* by ethyl acetate extraction, silica gel chromatography and HPLC. Resorthiomycin exhibited an *in vitro* cytotoxic activity against mouse leukemia L5178Y cells (IC_{50}, 15.5 μg/ml) and also inhibited the clonogenic activity of a multidrug-resistant mutant of human hepatoma PLC/PRF/5 cells to a greater extent than that of the parental cells. On the other hand, this antibiotic does not possess any antibacterial or antifungal activity.

The emergence of cancer cells acquiring resistance to anticancer drugs is one of the most serious problem in cancer chemotherapy. Thus, we have been searching for new microbial products with a selective toxicity against such resistant cancer cells and found that a soil actinomycete strain 45H-6 produces a novel antibiotic, to which multidrug-resistant cells of human hepatoma PLC/PRF/5 are more sensitive than the parental cells. The new antibiotic was designated resorthiomycin after its chemical characteristics[1]. Taxonomy of the producing organism, isolation and some biological activities of this antibiotic are presented in this publication.

Taxonomy of the Producing Organism

The producing organism, strain 45H-6, was isolated in our laboratory from a soil sample, collected at Isehara, Kanagawa Prefecture, Japan. Characterization of the strain principally followed the methods adopted by the International Streptomyces Project (ISP)[2].

Morphological and Chemical Properties

Microscopic studies showed that fairly long, straight or wavy aerial mycelia arose from well branched, non-fragmented substrate mycelia when grown on most agar media. Spore chains were formed on short sporophores branching monopodially from aerial hyphae. The chains appeared as tight terminal spirals approximately 4 μm in diameter with a few (1~3) turns, and tight coils with more frequent (6~9) turns were often observed in heavily grown parts of aerial mycelia. Most chains of mature spores contained 10 to 50 or more spores per chain, but occasionally there were shorter chains looking like loops or hooks in poorly grown parts of aerial mycelia (Plate 1). The spores were cylindrical (0.5~0.6 × 1.0~1.2 μm) with a smooth surface as seen by the electron microscope (Plate 2). As special morphology, moisture droplets frequently observed around spirals and then coalesced into a masses of spores originating at the spiral

Plate 1. Tight spiral appearance of spore chains of strain 45H-6 which were formed on aerial mycelia when cultivated on sucrose - nitrate agar for 14 days at 27°C.

Bar represents 20 μm.

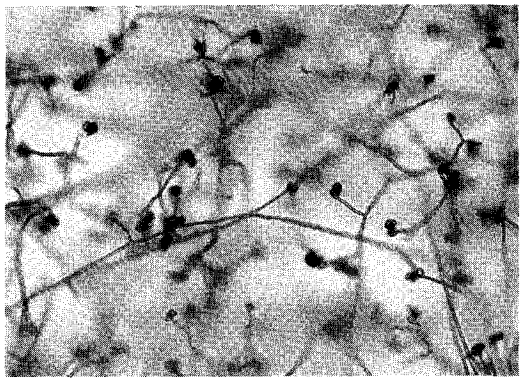


Plate 2. An electron micrograph of spore chains of strain 45H-6 which was cultivated on oatmeal agar for 10 days at 27°C.

Bar represents 1 μm.

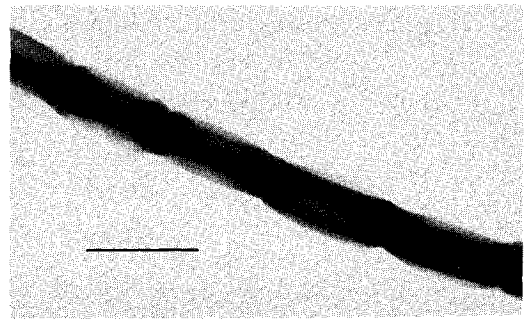


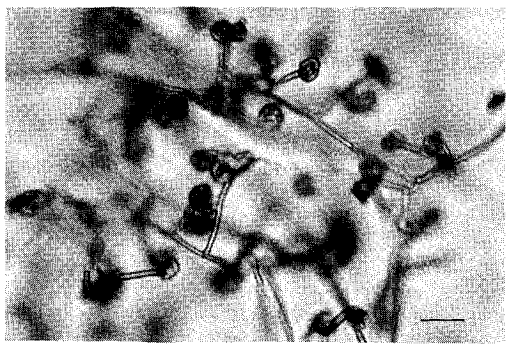
Plate 3. Special morphology of strain 45H-6 cultivated for 14 days at 27°C.

(A) Terminal spirals and coalesced spore-masses observed on sucrose - nitrate agar.

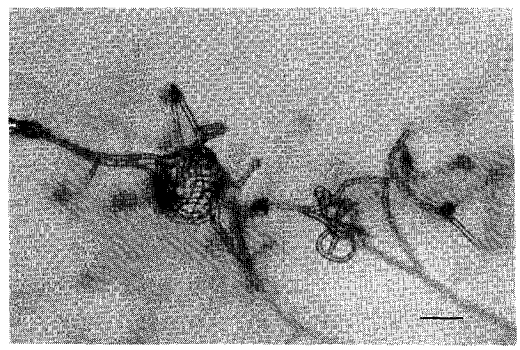
(B) Knots of hyphae developing into nest-like body on aerial mycelium observed yeast extract - malt extract agar.

Bars represent 1 μm.

(A)



(B)



parts (Plate 3A). Moreover, knots of hyphae and nest-like bodies in aerial mycelia frequently developed on most media (Plate 3B).

Chemical analysis of whole cell hydrolysates by the method of BECKER *et al.*[3] showed the presence of LL-diaminopimelic acid. It indicates that the cell wall type of this strain is type I.

Cultural Characteristics

Cultural studies were performed with cultures grown at 27°C on the 3, 7, 14 and 21 days of incubation. The colors recorded for mature cultures were determined according to the "Color Harmony Manual"[4].

The cultural characteristics observed on various media are summarized in Table 1. Mature aerial mycelia corresponded to both the gray and the red color series[5], which could be described as light brownish gray. The reverse side of the colony was brown or not distinctively pigmented, and the color was not changed by pH of the medium. Melanoid pigments were produced on tyrosine agar, Tryptone - yeast

broth, and peptone - yeast extract - iron agar. Brownish diffusible pigments (no pH indicator) were formed on some media.

Physiological Studies

The organisms were grown in a temperature range of 20～40°C (optimum 27～30°C) on oatmeal agar. The utilization of carbon compounds was examined by the method of PRIDHAM and GOTTLIEB[6]. Growth of the strain was supported by the following carbohydrates as a sole carbon source: D-Glucose, D-xylose, L-arabinose, L-rhamnose, D-fructose, raffinose, D-mannitol, *i*-inositol, and salicin. Sucrose was not utilized under the same experimental conditions. Other physiological characteristics of the strain are summarized in Table 2.

Taxonomic Position

The strain 45H-6 was demonstrated to belong to the genus *Streptomyces* on the basis of findings that its cell wall type is type I and it has spore chains consisting of more than 10 spores per chain formed on

Table 1. Cultural characteristics of strain 45H-6.

Medium	Growth	Aerial mycelium	Reverse color	Soluble pigment
Sucrose - nitrate agar	Poor	Moderate, Red (light brownish gray, 5cb)	Colorless with aerial mass color	None
Glucose - asparagine agar	Moderate	Good, Red (light brownish gray, 5dc) or Gray (light brownish gray, 5fe)	Pale brown to brown (4qc-4pi)	Pale yellowish brown (3qc)
Glycerol - asparagine agar (ISP medium 5)	Moderate	Poor, whitish to Gray (light brownish gray, 3fe)	Pale or light brown (5ie-5le)	Pale orange (4ea)
Inorganic salts - starch agar (ISP medium 4)	Good	Good, Gray (light brownish gray, 3fe-5fe)	Light brown to light brownish gray (4qe-4ie)	Light brownish gray (3ec)
Tyrosine agar (ISP medium 7)	Moderate	Good, Gray (gray, e)	Dark yellowish brown (3pl)	None
Nutrient agar	Poor	None	Colorless	None
Yeast extract - malt extract agar (ISP medium 2)	Good	Good, Gray (light brownish gray, 2fe-3fe)	Light brown (4ie-4lg)	None
Oatmeal agar (ISP medium 3)	Good	Good, Red (light brownish gray, 5dc-5ge)	Light brown to light brownish gray (4qe-4ne)	Pale yellowish brown (3ie)

Incubation at 27°C for 2 weeks.
Red: Red color series, Gray: gray color series.
( ): Color code designations followed the color standard[4].

Table 2. Physiological properties of strain 45H-6.

Property	Medium	Response
Starch hydrolysis	Inorganic salts - starch agar	+
Gelatin liquefaction	Glucose - peptone - gelatin	+
Milk coagulation	Skim milk	−
Milk peptonization	Skim milk	+
Melanoid pigment production	Tryptone - yeast broth	+
	Tyrosine agar	+
	Peptone - yeast extract - iron agar	+

Table 3. Comparison of strain 45H-6 with *Streptomyces collinus*.

Characteristics	Strain 45H-6	*S. collinus*
Spore chain morphology	Tight spirals, few turns or many turns of small diameter (loops, hooks)	Tight spiral, few turns of small diameter (loops, hooks)
Spore numbers per chain	10 to 50 or more (short)	3 to 10 (long)
Coalescent masses of spores	+	+
Spore surface	Smooth	Smooth
Color of colony	Gray or red color series	Gray (or red) color series
Reverse side of colony	Pale or light brown or no distinct pigment	No distinct pigment
Production of melanoid pigment on:		
PYIA	+	+
TA	+	−
TYB	+	− (+)
Other soluble pigments	None or trace of brownish pigments	None
Utilization of sucrose	−	+

PYIA: Peptone-yeast extract-iron agar, TA: Tyrosine agar, TYB: Tryptone-yeast broth.
+: Positive, −: negative.
( ): Occasionally observed.

aerial hyphae.

Among the known *Streptomyces* species listed in "Approved Lists of Bacterial Names"[7,8] and other validation lists of bacterial names[9], the strain closely resembles *Streptomyces collinus*. The morphological and physiological characteristics of the strain were compared with those of *S. collinus*[10] and, as a result, good agreement was obtained between the two strains except for utilization of sucrose and production of melanoid pigments in tyrosine agar (Table 3). The difference in these properties are not sufficient to classify the strain as a distinct species. Thus, strain 45H-6 should be tentatively placed in the species *S. collinus* Lindenbein.

Fig. 1. Purification procedure for resorthiomycin.

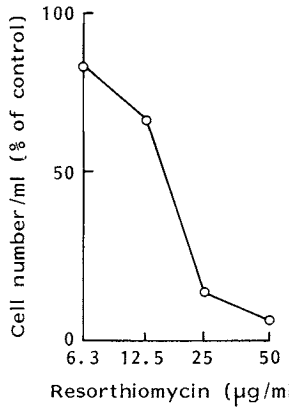
Broth filtrate (60 liters)
↓ adjusted to pH 3, extracted with EtOAc (100 liters)
EtOAc layer
↓ concd *in vacuo*
Silica gel column chromatography
↓ developed with $CHCl_3$-MeOH (200:1)
Active fractions
↓ concd *in vacuo*
ODS reverse-phase HPLC
↓ eluted with 65% CH_3CN
Resorthiomycin (39.1 mg)

Production and Isolation of Resorthiomycin

The procedure for purification of resorthiomycin is illustrated in Fig. 1. The vegetative inoculum was obtained by transferring spores of strain 45H-6 to a 500-ml Sakaguchi flask containing 100 ml of a medium consisting of oatmeal 2% and yeast extract 0.1%, pH 7.2. The flasks were incubated at 27°C for 72 hours on a reciprocal shaker (150 rpm). For jar fermentation, 600 ml of inoculum was added to 30 liters of the same production medium as that used for shake flask fermentation in a 40-liter jar fermenter, which was stirred at an impeller speed of 200 rpm and aerated at 10 liters per minute. The fermentation was terminated after approximately 120 hours of incubation at 27°C.

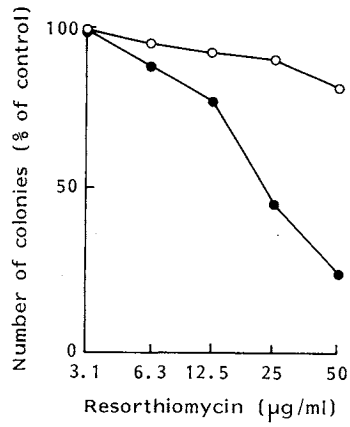
Antibiotic in the culture fluid (60 liters) was extracted with ethyl acetate (100 liters) at pH 3. The ethyl acetate layer was then concentrated to dryness. The residue was dissolved in a small volume of chloroform,

Fig. 2. Inhibition by resorthiomycin of growth of mouse leukemia L5178Y cells.



L5178Y cells (1.5×10^4 cells/ml) in RPMI-1640 medium supplemented with 10% fetal calf serum were cultured at 37°C for 3 days in the presence of various concentrations of resorthiomycin. Number of cells were determined with a Coulter counter and expressed as percentage of control. Each point represents the mean of triplicate determinations.

Fig. 3. Effect of resorthiomycin on the colony formation of the parental strain (PLC/S) (○) and a multidrug-resistant mutant (PLC/COL) (●) of human hepatoma PLC/PRF/5 cells.



Cells (200～300/dish) were suspended in EAGLE's minimum essential medium supplemented with 10% calf serum and plated on 60-mm plastic dishes. Resorthiomycin was added 18 hours after inoculation and the number of colonies in duplicate cultures was counted after 2～3 weeks and expressed as a percentage of control.

and the solution was applied to a silica gel column (2.5×40 cm), and developed with chloroform-methanol (200:1). The active fractions were collected and concentrated to dryness, and further purified by ODS reverse-phase HPLC. Elution with 65% acetonitrile yielded 39.1 mg of resorthiomycin.

Biological Activity of Resorthiomycin

Resorthiomycin exhibited neither antibacterial nor antifungal activity at a concentration of 100 μg/ml, while it inhibited dose-dependently growth of mouse leukemia L5178Y cells with a IC_{50} of 15.5 μg/ml (Fig. 2). Moreover, resorthiomycin suppressed the colony formation of a multidrug-resistant human hepatoma PLC/PRF/5 cell line (PLC/COL), which has been established in our laboratory by cultivating cells in a medium containing colchicine, to a greater extent, than that of the parental cell line (PLC/S) (Fig. 3). Because difference in number of colonies in duplicate plates was less than 10%, the values of PLC/S and PLC/COL with resorthiomycin at 25 and 50 μg/ml are significantly different. PLC/COL, a typical phenotype of multidrug resistance which is resistant to a wide range of structurally unrelated antitumor drugs, showed a collateral sensitivity to resorthiomycin (Table 4).

Table 4. Effects of resorthiomycin and other antitumor drugs on the colony formation of human hepatoma PLC/S and PLC/COL cells.

Drug	IC_{50}		Degree of resistance[a]
	PLC/S	PLC/COL	
Resorthiomycin	>50 μg/ml	24 μg/ml	<0.5
Actinomycin D	2.6 ng/ml	23 ng/ml	9
Doxorubicin	9.9	97	10
Colchicine	3.0	130	45
Vincristine	1.4	36	26

[a] The degree of resistance was expressed as ratio of IC_{50} values for resistant (PLC/COL) to parental (PLC/S) cell line.

Discussion

One of the major problems in the treatment of cancer is the development of resistance to anticancer

agents. We have been screening new antitumor antibiotics active against drug-resistant tumor cells and, as a result, lactoquinomycin was discovered, which inhibits *in vitro* growth of the drug-resistant sublines of L5178Y mouse leukemia cells more profoundly than that of the parental cell line[11~13]. In the course of a similar screening program using the multidrug-resistant tumor cell line established in this laboratory, we discovered resorthiomycin from the culture broth of a strain of *S. collinus*. Resorthiomycin has a unique structure possessing a 6-substituted benzene ring in the molecule as described in the accompanying paper[1] and preferentially inhibited *in vitro* growth of multidrug-resistant human hepatoma PLC/PRF/5 cells. The mode of action of resorthiomycin is reported in a subsequent paper[14]. The efficacy of resorthiomycin in experimental animal tumor systems remains to be studied.

Acknowledgments

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