CC-1065 (NSC-298223), A NEW ANTITUMOR ANTIBIOTIC

PRODUCTION, *IN VITRO* BIOLOGICAL ACTIVITY, MICROBIOLOGICAL ASSAYS AND TAXONOMY OF THE PRODUCING MICROORGANISM*

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A new antitumor antibiotic is produced in fermentation liquors of *Streptomyces zelensis* sp.n. The antibiotic is biologically active at extremely low concentrations. At 40 pg/ml, it inhibited 90% of the growth of L1210 cells in culture in tube dilution assays. The minimal inhibitory concentrations against Gram-positive bacteria is between 1~10 ng/ml, while these values for Gram-negative bacteria and fungi are mostly under 1 μg/ml. A microbiological assay with *Bacillus subtilis* can detect concentrations of 1~2 ng/ml.

A new antitumor antibiotic, CC-1065, was discovered in our laboratories[1]. It was isolated from fermentation liquors of a new species of *Streptomyces* which was designated *Streptomyces zelensis* DIETZ and LI sp. n. (UC®-5923).

This communication describes the production of the drug, the taxonomic study of the producing microorganism, the *in vitro* activity, and the microbiological assay.

The methods of isolation and evaluation against experimental animal tumors will be described in separate communications.

Materials and Methods

Taxonomy

A new species of *Streptomyces*, isolated from soil, has been characterized as *Streptomyces zelensis* DIETZ and LI sp.n. The methods used may be found in DIETZ,[2,3]** DIETZ and MATHEWS,[4] and SHIRLING and GOTTLIEB[5].

Production

Stock cultures of *Streptomyces zelensis* were frozen plugs prepared from heavy surface growth on agar and kept in a liquid nitrogen storage tank. The seed medium contained 5 g of Bacto-Tryptone (Difco Laboratories, Detroit, Mich.), 3 g of yeast extract (Difco) and 1 g of dextrin (A. E. Staley Mfg., Decatur, Ill.) per liter of deionized water. It was inoculated with the stock culture and incubated on a 250-rpm rotary shaker for 48 hours at 28°C. The seed was used to inoculate the production media at a rate of 5% (v/v).

The production media contained 10 g black strap molasses (Knappen-Milling Co., Augusta, Mich.), 10 g of dextrin, 10 g of N-Z Amine Type A (Sheffield Chemical Co., Norwich, N. Y.), and 5 g calcium carbonate per liter of tap H_2O (pH 7.2 prior to autoclaving). The fermentation was carried out in 500-

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** Modified. Reference color from ISCC-NBS Color Names Chart for NBS Circular 553 only. The Color Harmony Manual is no longer available.

ml non-stippled flasks (each containing 100 ml) at 28°C on a rotary shaker (250 rpm) for 120 hours.

In Vitro Evaluation

Broad antimicrobial testing of CC-1065 was done by determining the minimal inhibitory concentrations (MIC) in suitable liquid media following 42-hour incubation. After that the two lowest non-growing tubes were subcultured to determine whether the effect of the drug was bactericidal or just bacteriostatic.

The *in vitro* activity against L1210 mouse leukemia cells growing in suspension culture was determined using the tube dilution technique described by BUSKIRK[6].

Antimicrobial Assay

Strong antimicrobial activity in liquid media was utilized to develop sensitive turbidimetric assay for CC-1065. For one assay we utilized *Micrococcus luteus* (*Sarcina lutea*) UC-130 cultivated in brain heart infusion broth (Difco) on a reciprocating shaker at 37°C for 16~18 hours. The extent of growth was then determined by measuring the optical density with a Beckman spectrophotometer at 600 nm. Because of low water solubility, CC-1065 was first dissolved in methylene chloride and further diluted to 5 μg/ml in solution containing 50% acetone and 50% water. This stock solution was stable for several months at −20°C. Before assay the standard solutions of pure CC-1065 were prepared by further diluting the stock solutions in brain heart infusion to 50 ng/ml. Concentrations suitable for the construction of standard curve were between 50 and 2 ng/ml. Usually a 1.5 dilution scale was used. For a more highly sensitive assay that can be read after 2~2.5 hours of incubation, we use spores of *Bacillus subtilis* (UC-564) that have been germinated for 2 hours in brain heart infusion broth at 37°C. Since the growth of *B. subtilis* is poorly suitable for accurate turbidimetric readings, it can only be estimated by visual observations. However, its speed makes it a valuable tool in situations when fast results—even though less accurate— are desirable.

Assay Using L1210 Cells in Culture

The inhibition of L1210 cells in culture was used as the basis for a quantitative assay. The details of this method have been described before[6]. It is a very sensitive technique; the only disadvantage is that it takes 3 days to complete. The concentrations used for standard curves were between 3~100 pg/ml.

Results and Discussion

Taxonomy

Color Characteristics: Aerial growth green-gray or gray-green. Melanin negative. The appearance of the culture on Ektachrome is given in Table 1. Reference color characteristics are given in Table 2. The culture may be placed in the yellow (Y) and green (GN) color series of TRESNER and BACKUS[7].

Table 1. Appearance of *Streptomyces zelensis* on Ektachrome[2,3]

Agar medium	Surface	Reverse
BENNETT's	Trace gray	Yellow-tan
CZAPEK's sucrose	Pale gray	Colorless
Maltose-tryptone	Gray-green	Tan
Peptone-iron	Gray-white	Tan
0.1% Tyrosine	Trace gray	Pale yellow-tan
Casein starch	Pale gray-green	Pale tan

Microscopic Characteristics: Spore chains at first straight, then becoming open spiral to spiral (RF, RA, S in the sense of PRIDHAM *et al.*)[8]

Cultural and Biochemical Characteristics: Cultural and biochemical characteristics are described in Table 3.

Carbon Utilization: Growth on carbon compounds was determined using the procedures of PRIDHAM and GOTTLIEB[9] and SHIRLING and GOTTLIEB.[5] In the former the culture grew well on D-xylose, D-fructose, D-galactose, D-glucose, D-mannose, maltose, cellobiose, dextrin, soluble starch, glycerol, D-mannitol, and inositol; moderately on L-arabinose, sodium oxalate, sodium acetate, and

Table 2. Reference color characteristics of *Streptomyces zelensis*

	Determination	ISCC-NBS Centroid color charts standard sample No. 2106 [Supplement to NBS circular 553[22]]	
		Color chip	Color name
BENNETT'S	S	121 p. YG	Pale yellow green
	R	86 l. Y	Light yellow
	P	76 l. yBr	Light yellowish brown
CZAPEK's sucrose	S	121 p. YG	Pale yellow green
	R	92 y White	Yellowish white
	P	—	
Maltose-tryptone	S	122 gy. YG	Grayish yellow green
	R	91 d. gy. Y	Dark grayish yellow
	P	76 l. yBr	Light yellowish brown
HICKEY-TRESNER	S	122 gy. YG	Grayish yellow green
	R	90 gy. Y	Grayish yellow
	P	76 l. yBr	Light yellowish brown
Yeast extract-malt extract (ISP-2)	S	122 gy. YG	Grayish yellow green
	R	91 d. gy. Y	Dark grayish yellow
	P	71 m. yBr	Moderate yellowish brown
Oatmeal (ISP-3)	S	121 p. YG	Pale yellow green
	R	104 p. gy. Y	Pale grayish yellow
	P	—	
Inorganic salts-starch (ISP-4)	S	122 gy. YG	Grayish yellow green
	R	105 gy. gY	Grayish greenish yellow
	P	76 l. yBr	Light yellowish brown
Glycerol-asparagine (ISP-5)	S	121 p. YG	Pale yellow green
	R	90 gy. Y	Grayish yellow
	P	—	

S=Surface R=Reverse P=Pigment

sodium succinate; and poorly on rhamnose, sucrose, lactose, raffinose, inulin, dulcitol, D-surbitol, salicin, phenol, sodium formate, sodium tartrate, sodium salicylate, sodium citrate, and the control (no carbon compound added). The culture did not grow on cresol. In the latter the culture grew well on the positive control (D-glucose), D-xylose, inositol, D-mannitol, and D-fructose; moderately on L-arabinose and poorly on the negative control (basal medium). It did not grow on sucrose, rhamnose, raffinose, or cellulose.

Temperature: The culture grew slightly at 4°C, moderately at 18°C, 24°C, and 45°C, and well at 28°C, 32°C, and 37°C. There was no growth at 55°C. Media used for temperature studies were BENNETT's, CZAPEK's sucrose, maltose-tryptone, and HICKEY-TRESNER agars.

Antibiotic-producing Properties: The culture produces the antitumor agent CC–1065 (NSC–298223).

Source: Soil.

Type Culture: *Streptomyces zelensis* sp. n. UC®-5923.

A new soil isolate, which produces the antitumor activity CC-1065 is characterized and considered to be a new species of the genus *Streptomyces* on the basis of its conformity to the general characteristics of the genus.[10]

This new isolate has gray-green aerial growth, is melanin-negative, has short, straight to open spiral to spiral spore chains of round spores with a spiny or thorny surface. The culture can be differentiated

Table 3. Cultural and biochemical characteristics of *Streptomyces zelensis*

	Medium	Surface	Reverse	Other characteristics
Agar media	Peptone-iron	Pale gray-green	Tan	Tan pigment Melanin negative
	Calcium malate	Pale gray-green	Olive	No pigment Malate not solubilized
	Glucose asparagine	Pale gray-green	Pale chartreuse	No pigment
	Skim milk	Trace pale gray-pink	Orange-tan	Orange-tan pigment Casein solubilized
	Tyrosine	Green-gray	Brown	Light brown pigment Tyrosine solubilized
	Xanthine	Green-gray	Light tan	Light tan pigment Xanthine not solubilized
	Nutrient starch	Green-gray	Yellow-green-tan	Light tan Starch hydrolyzed
	Yeast extract-malt extract	Green	Tan	Tan pigment
	Peptone-yeast extract-iron (ISP-6)	Pale gray-white	Tan	Tan pigment Melanin negative
	Tyrosine (ISP-7)	Gray-green	Gray	No pigment Melanin negative
Gelatin media	Plain	White aerial growth on surface pellicle	—	Yellow-tan pigment Complete liquefaction
	Nutrient	White aerial growth on surface pellicle	—	Yellow-tan pigment Complete liquefaction
Broth media	Synthetic nitrate	—	—	Very slight bottom growth Nitrate reduced to nitrite
	Nutrient nitrate	Green-white aerial growth on surface pellicle		Yellow to yellow tan pigment Very slight bottom growth Nitrate reduced to nitrite
	Litmus milk	Gray-green-white aerial growth on surface pellicle		Blue surface pigment in two of 3 tubes Tan pigment Peptonization pH 7.9

from members of the limited "green" group,* of *Streptomyces* by its color pattern on Ektachrome, its growth on carbon compounds in synthetic media, and by the production of the antitumor agent CC-1065.

Therefore, it is proposed that the new soil isolate be designated *Streptomyces zelensis*** DIETZ and LI sp. n. and that this type species be designated the type subspecies *Streptomyces*

Table 4. Production of CC-1065 by *S. zelensis* in 500-ml flasks

Time of incubation (hours)	pH	Cytotoxic equivalents of CC-1065 (μg/ml)
48	7.7	1.4
72	8.3	3.3
96	8.6	2.4
120	8.6	1.6

* Green group cultures are those in the *Viridis* Series of WAKSMAN[12] and BALDACCI[13]; the *prasinus* color group of ETTLINGER *et al.*[14]; the *prasinus* odor *azureus-glaucus* group of HÜTTER[15], the *malachiticus* group of KÜSTER[16]; the green-spore color group X of KUTZNER[17], the prasinomycin-producers of MYERS *et al.*[18,19], those in Tables 11 and 12 in KÜSTER[20], and those in the green series of PRIDHAM and TRESNER[21].

** Derived from Zeleny=green in Czech.

Table 5. *In vitro* antimicrobial spectrum of CC-1065 in liquid media

	Microorganisms	42-hour MIC (μg/ml)
Gram-positive bacteria	*Staphylococcus aureus* UC-76	0.0015 (cidal)
	Staphylococcus aureus UC-552	0.003 (cidal)
	Staphylococcus aureus UC-70	0.0015 (cidal)
	Staphylococcus aureus UC-3665	0.003 (cidal)
	Bacillus subtilis UC-564	0.012 (cidal at 0.025)
	Streptococcus pyogenes UC-6055	0.0008 (cidal)
	Micrococcus luteus (*Sarcina lutea*) UC-130	0.012 (cidal)
	Streptococcus faecalis UC-157	0.012 (cidal)
	Streptococcus faecalis UC-3235	0.003 (cidal)
Gram-negative bacteria	*Escherichia coli* UC-51	0.32 (cidal)
	Proteus vulgaris UC-93	0.08 (cidal)
	Pseudomonas aeruginosa UC-95	0.08 (cidal)
	Pseudomonas putida UC-3029	0.15 (cidal at 0.3)
	Salmonella gallinarum UC-265	2.5 (cidal)
	Klebsiella pneumoniae UC-57	0.08 (cidal)
	Salmonella schottmuelleri UC-126	0.3 (cidal)
	Salmonella sp. Serotype *pullorum* UC-267	0.08 (cidal)
Fungi	*Candida albicans* UC-1392	0.3 (cidal)
	Saccharomyces cerevisiae UC-1337	0.04 (cidal)
	Saccharomyces cerevisiae UC-1342	0.3 (cidal)
	Penicillium oxalicum UC-1268	0.02 (cidal)

Table 6. A three-hour turbidimetric assay of CC-1065 with *B. subtilis*

Concentration of CC-1065 (ng/ml)	Growth
25	0
16.67	0
11.11	0
7.41	0
4.94	0
3.29	0
2.19	1
1.46	1
0.97	2
0.65	2
0 (control)	2

zelensis subsp. *zelensis* in accordance with the rules set forth in the International Code of Nomenclature of Bacteria[11].

Production

The results of a typical fermentation carried out in 500-ml plain fermentation flasks are presented in Table 4. The peak titers were usually reached after 3 days of incubation and the titers were estimated by a microbiological tube-dilution assay with *M. luteus* or by an assay based on inhibition of L1210 cells in culture.

In Vitro Evaluation

(1) Antimicrobial Effect

The minimal inhibitory concentrations(MIC) of CC-1065 against a spectrum of selected microorganisms is presented in Table 5. Very low inhibitory concentrations after 42 hours incubation were recorded and almost all these levels were bactericidal.

(2) Cytotoxic Effect

The inhibition by CC-1065 of growth of mouse leukemia L1210 cells cultivated in liquid media is presented in Fig. 1. Only about 40 pg/ml was necessary for 90% inhibition of growth.

Antimicrobial Assay

A typical dose response of CC-1065 against *S. lutea* after 18 hours at 37°C is presented in Fig. 2.

The result of a short-time (2.5 hours) assay with *B. subtilis* spores is presented in Table 6.

From all the experimental results it appears that CC-1065 is among the most potent antitumor drugs ever discovered. Its growth inhibitory effects towards L1210 cells are observed at concentrations lower

Fig. 1. Inhibition by CC-1065 of growth of L1210 cells in culture

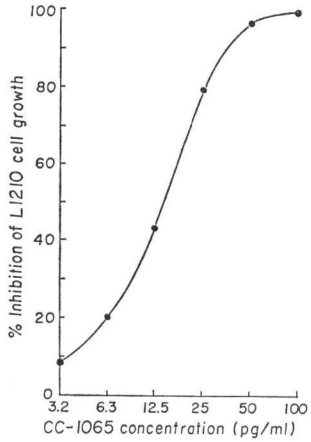
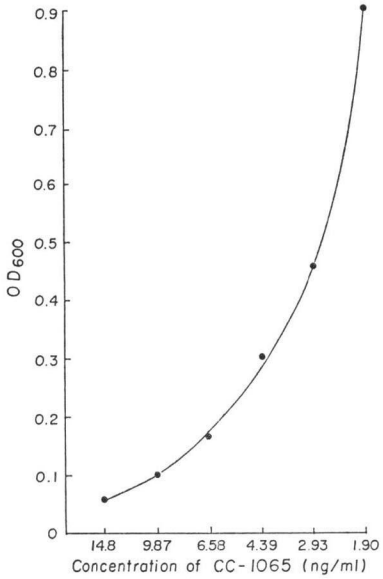


Fig. 2. Turbidimetric assay of CC-1065 with *S. lutea*



than any other antitumor agent tested in our laboratories.[23] At present the peak fermentation titers are only 2~3 μg/ml. However, very low doses are required to elicit an antitumor effect. For example, doses between 3~30 μg/kg resulted in significant increase in life span in mice inoculated with P388 or L1210 melanoma[1]. More extensive investigations on *in vivo* antitumor effect and on biochemical behavior of CC-1065 are in progress.

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