NOVEL ANTITUMOR AGENTS CI-920, PD 113,270 AND PD 113,271

I. TAXONOMY, FERMENTATION AND BIOLOGICAL PROPERTIES

J. B. TUNAC, B. D. GRAHAM and W. E. DOBSON

Warner-Lambert/Parke-Davis Pharmaceutical Research
Ann Arbor, MI 48105, USA

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CI-920 (PD 110,161) and two analogues (PD 113,270 and PD 113,271) are novel antitumor compounds produced by a new actinomycete characterized as *Streptomyces pulveraceus* subsp. *fostreus* ATCC 31906. The antitumor compounds are predominantly produced during the stationary (idiophase) growth phase of the organism.

CI-920 is active *versus* the murine P388 lymphocytic and L1210 lymphoid leukemia with T/C values of 246 and 207, respectively. This compound has no significant antimicrobial activity.

In our screening program for antitumor compounds, a fermentation broth of an actinomycete (isolate WP-426) was observed to inhibit the growth of L1210 murine leukemia cells in tissue culture. The biological activity was subsequently found to be due to a complex of at least three antitumor compounds: CI-920 (component A), PD 113,270 (component B), and PD 113,271 (component T). The isolation and characterization of these phosphorus-containing compounds are described in the following paper.[1] In this paper, the taxonomy of the producing organism, fermentation, and biological properties of the compounds are presented.

Materials and Methods

Culture Characterization

The culture was isolated from a soil sample pretreated with calcium carbonate.[2] The soil was subsequently plated on to an agar medium consisting of the following ingredients: glycerol 3%, L-asparagine 0.25%, potassium chloride 0.05%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001%, and agar 1.5%.

Culture characterization was carried out following the ISP (International Streptomyces Project) procedure.[3] In addition, Waksman starch agar (WSA)[4] and Amidex corn starch agar (ADX)[5] were used. The culture was maintained on ADX agar slants at 28°C. Morphological and color determinations of the growth of the organism were made at weekly intervals over a three-week period. Cell wall analysis was carried out following the methods of BECKER *et al.*[6]

Fermentation

Stock cultures of the organism were maintained in lyophilized vials and the working culture on ADX agar slants. The microbial growth from a two-week old slant was used to inoculate a 300-ml seed flask and incubated with shaking (New Brunswick Shaker, 5-cm throw) at 24°C. The seed medium consisted of: 0.5% Amberex 1003 (Amber Labs), 0.1% Cerelose, 2.4% dextrin Amidex B411 (Corn Products), 0.5% N-Z Case (Humko-Sheffield), 0.3% spray dried meat solubles (Daylin Labs), and 0.2% $CaCO_3$. The production of the antitumor complex was carried out in 300-ml shake-flasks, 30-liter stirred-jars, or in 760-liter fermentors. The production medium consisted of: 5% glycerol, 0.5% spray-dried meat solubles, 0.5% distillers solubles, 0.25% blood protein hydrolysate, 0.2% torula yeast, 0.1% NaCl, 0.25% $CaCO_3$, 0.25% KH_2PO_4. The fermentation conditions were as follows: shake-flask, 50 ml/300-ml flask, 200 rpm shaker (Model G-53, New Brunswick Shaker); 30-liter stirred-jar

fermentor, 16 liters/jar, 0.75 v/v/minute, 300 rpm; 760-liter fermentor, 600 liters/tank, 0.75 v/v/minute (570 liters/minute), 155 rpm. The fermentation was carried out for 4~6 days at 24°C.

Assay and Antitumor Activity

The antitumor complex in the fermentation beer was assayed both by high pressure liquid chromatography (HPLC)[1] and activity *versus* L1210 murine leukemia cells (Mason Res. Inst., Worchester, Massachusetts) in tissue culture. The *in vivo* antitumor activity was evaluated *versus* P388 murine lymphocytic leukemia and L1210 murine lymphoid leukemia tumor cell lines in CDF_1 mice.[7] The tumor cells were injected intraperitoneally (i.p.) on day 0 and the compound was also introduced i.p. on days 1 through 9.

Cell Wall Analysis

The major components of the cell wall of isolate WP-426 were found to consist of glucosamine, muramic acid, alanine, and glutamic acid. In addition, glycine and LL-α,ε-diaminopimelic acid were observed. The above cell wall analysis indicates that the isolate belongs to the "Type 1" or "*Streptomyces*-type" actinomycete.[6]

Results

Morphological and Cultural Characteristics

Results of preliminary cultural characterization indicated that isolate WP-426 is similar to *Streptomyces pulveraceus*. Thus, a systematic comparison was carried out between isolate WP-426 and the reference culture, *S. pulveraceus* ATCC 13875. The morphology and color of the two organisms varied when grown on different media. The reference culture produced predominantly spiral spores on all media used, while WP-426 produced predominantly spiral spores on ISP media 3 and 4, and WSA; flexibilis spores on ISP medium 2 and ADX; and rectus spores on ISP medium 5 (Table 1). The characteristic spore chain morphology of WP-426 as compared to *S. pulveraceus* ATCC 13875 is shown in Fig. 1. The morphology of the spore-bearing hyphae for the two organisms is shown in Fig. 2.

Physiological Characteristic

Isolate WP-426 was found to produce melanin, liquefies gelatin, and utilizes the tested carbon sources except cellulose, inositol, and mannitol (Table 2).

The WP-426 isolate differs from the reference *S. pulveraceus* ATCC 13875 in that the latter does not produce pigments and does not utilize arabinose.

Fermentation and Production of CI-920

The fermentation medium and process developed in shake-flasks for the production of the antitumor complex was successfully scaled up into 30-liter stirred-jars and in a 760-liter fermentor. A typical fermentation pattern which includes the growth (% sedimentation), dissolved oxygen (DO_2), and pH in relation to the production of the compound is shown in Fig. 3. Production of the antitumor complex starts about 24 hours after inoculation. Maximum yields were obtained after 120 hours of fermentation: the yields of the A and T components are about the same, 400~430 μg/ml range; while the B component was produced in very low level, <30 μg/ml. Good growth is favored above pH 5.5, whereas maximum production of the antitumor complex occurs below pH 5.5. Thus, the initial pH of 6.2 is conducive to rapid microbial growth during the first 35 hours at which time the pH drops to 5.5. The growth rate then slows down while the rate of antibiotic production increases. At about 100 hours the pH starts to rise; good growth resumes, and antibiotic production levels off.

The similarity in cultural and physiological characteristics of WP-426 to *S. pulveraceus* prompted

Table 1. Cultural characteristics of *S. pulveraceus* subsp. *fostreus* ATCC 31906, and *S. pulveraceus* ATCC 13875.

Medium	Color and sporulation		ATCC 31906	ATCC 13875
Yeast extract - malt extract agar (ISP Medium 2)	Color[1]	AM	Beige gray (3 ih)	Lead gray (5 ih)
		R	Dark brown (4 pn)	Deep brown (3 pl)
		SP	Amber (3 pe)	Oak brown (4 pi)
	Spore chain[2] (type and percent)	S	20	60
		F	60	0
		R	20	40
Oatmeal agar (ISP Medium 3)	Color	AM	Beige gray (3 ih)	Silver gray (5 fe)
		R	Silver gray (3 fe)	Marigold (3 la)
		SP	Light beige (3 ec)	Bamboo (2 gc)
	Spore chain (type and percent)	S	90	90
		F	10	10
		R	0	0
Inorganic salts - starch agar (ISP Medium 4)	Color	AM	Lead gray (5 ih)	Camel (3 ge)
		R	Ebony (3 po)	Dark brown (4 pn)
		SP	None	Bamboo (2 gc)
	Spore chain (type and percent)	S	90	80
		F	10	10
		R	0	10
Glycerol - asparagine agar (ISP Medium 5)	Color	AM	Gray (e)	Gray (g)
		R	Dark brown (6 pn)	Cocoa brown (5 ni)
		SP	Tile red (5 ne)	Yellow maple (3 ng)
	Spore chain (type and percent)	S	10	50
		F	30	30
		R	60	20
Amidex corn starch agar	Color	AM	Beige gray (3 ih)	Silver gray (3 fe)
		R	Dark brown (6 pn)	Red mahogany (6½ pl)
		SP	Cinnamon (3 le)	Cinnamon (3 le)
	Spore chain (type and percent)	S	20	90
		F	60	10
		R	20	0
Waksman starch agar	Color	AM	Beaver (4 li)	Silver gray (3 fe)
		R	Lead gray (5 ih)	Honey gold (2 ic)
		SP	90	90
	Spore chain (type and percent)	S	90	90
		F	10	5
		R	0	5

[1] Color designation from Color Harmony Manual, 4th Ed., Container Corporation of America, 1958. Color: AM, aerial mycelium; R, reverse substrate mycelium; and SP, soluble pigment.

[2] Spore chain: S, spiral; F, flexibilis; and R, rectus.

the evaluation and comparison of their fermentation products. The reference *S. pulveraceus* ATCC 13875 was reported to produce zygomycin, paromomycin, and cycloheximide.[9] We confirmed that the reference culture did produce paromomycin and cycloheximide but did not produce the CI-920 or its analogues using the reported media[8,10,11] or on the CI-920 medium. On the other hand, the WP-426 isolate did not produce paromomycin or cycloheximide using the reported fermentation media or on the CI-920 fermentation medium.

Fig. 1. Spore chain morphology of isolate WP-426 and *S. pulveraceus* ATCC 13875 (×5,000).

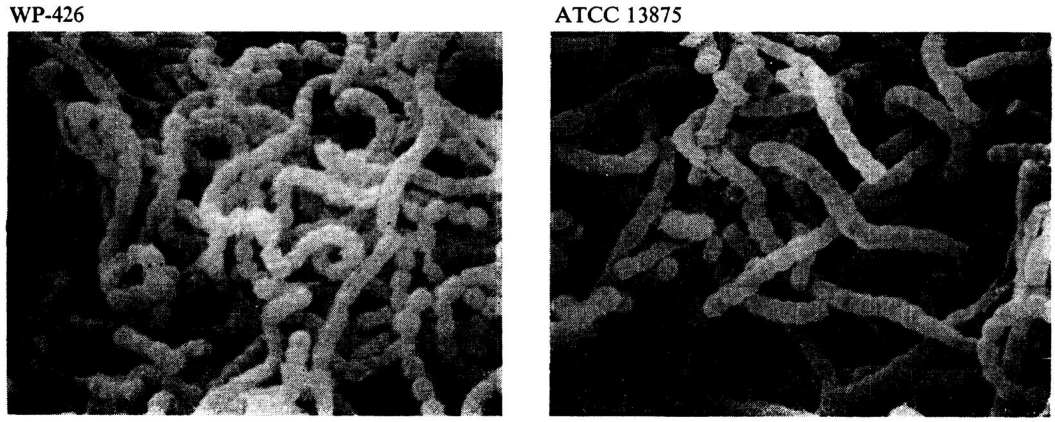


Fig. 2. Morphology of the spore-bearing hyphae of isolate WP-426 and *S. pulveraceus* ATCC 13875 (×250).

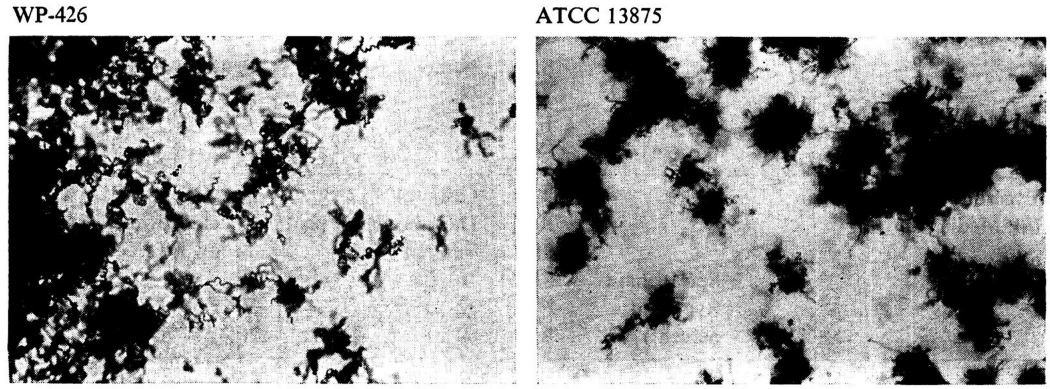
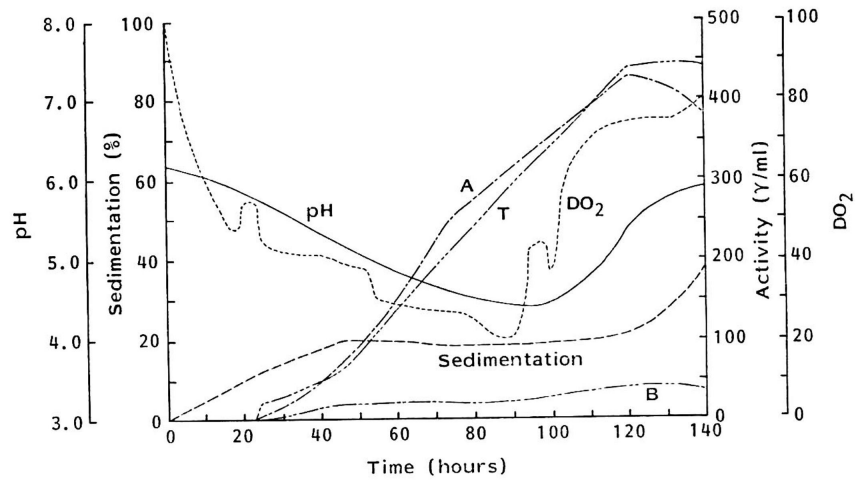


Fig. 3. Fermentation pattern of isolate WP-426 (*S. pulveraceus* subsp. *fostreus* ATCC 31906).



Biological Activity

The CI-920 and its analogs showed excellent antitumor activity in the murine tumor models. The maximum T/C values obtained *versus* P388 were 246, 178 and 175 for components A, B and T, respec-

Table 2. Cultural characteristics of *S. pulveraceus* subsp. *fostreus* ATCC 31906 and *S. pulveraceus* ATCC 13875.

	ATCC 31906	ATCC 13875
Melanin production on		
Tryptone - yeast extract broth (ISP Medium 1)	Positive	Negative
Peptone - yeast extract (ISP Medium 6)	Positive	Negative
Tyrosine agar (ISP Medium 7)	Slight	Negative
Gelatin liquefaction	Positive, brown soluble pigment	Positive, no soluble pigment
Skim milk coagulation	Negative, no soluble pigment	Negative, no soluble pigment
Nitrate reduction	Positive	Positive
Carbon utilization		
D-Glucose	+	+
L-Arabinose	+	−
Cellulose	−	−
D-Fructose	+	+
i-Inositol	−	−
Inulin	+	+
Maltose	+	+
D-Mannitol	−	−
Melibiose	+	+
Raffinose	+	+
Rhamnose	+	+
Sorbitol	−	−
Sucrose	−	−
D-Xylose	+	+
D-Galactose	+	+
Salicin	+	+
Control (no carbon)	−	−

Table 3. *In vivo* antitumor activity of CI-920 (component A) and its two analogs, PD 111,270 (component B) and PD 113,271 (component T) *versus* P388 lymphocytic and L1210 lymphoid leukemias.

Dosage[b]	T/C (%)[a]					
	P388			L1210[c]		
	Component A	Component B	Component T	Component A	Component B	Component T
25	Toxic	Toxic	175	Toxic	—	—
12.5	246	178	147	117	Toxic	—
6.25	216	142	142	207	120	Toxic
3.13	195	—	—	170	168	121
1.56	150	—	—	137	180	121
0.75	—	—	—	—	155	116

[a] T/C is the quotient (expressed in %) of the survival time of treated animals (T) and the survival time of control animals (C). T/C values of ≥125 and ≥130 for L1210 and P388, respectively, are considered active.

[b] mg/kg/injection, daily ×9 (i.p.).

[c] Data provided by NCI (Drug Evaluation Branch, Division of Cancer Treatment).

tively. The maximum T/C values *versus* L1210 were 207, 180 and 121 for component A, B and T, respectively (Table 3). A detailed description of the antitumor activity is reported in a separate paper.[12]

The ID_{50} value of CI-920 *versus* L1210 cells in tissue culture is 0.073 μg/ml.

CI-920 or its two analogues was devoid of antimicrobial activity when tested *versus* the following organisms at 500 μg/ml: *Alcaligenes viscolactis, Bacillus subtilis, Branhamella catarrhalis, Escherichia coli, Kloeckera brevis, Micrococcus luteus, Penicillium avellaneum, Proteus vulgaris, Staphylococcus aureus, Streptococcus faecalis* and *Xanthomonas phaseoli.*

Discussion

The organism, isolate WP-426, resembles *Streptomyces pulveraceus* ATCC 13875, with minor morphological and physiological differences: Isolate WP-426 produces melanoid pigments and utilizes arabinose while *S. pulveraceus* ATCC 13875 does not. For the above reasons, isolate WP-426 has been classified as a subspecies of *S. pulveraceus.* The subspecies name *fostreus* was given since the organism produces phosphorus-containing antitumor agents. The organism was deposited with the American Type Culture Collection Bank (Rockville, Maryland) and identified as *Streptomyces pulveraceus* subsp. *fostreus* ATCC 31906.

Initially, the organism produced a low level (~30 μg/ml) of the CI-920 compound but by fermentation development, the yield was increased to about 400 μg/ml. Production of the antitumor complex starts in the midlog growth phase (tropophase) and continues on through the stationary phase (idiophase). An interesting characteristic of the organism during fermentation was that after an extended stationary phase of growth, a second growth phase was observed. This is reminiscent of a diauxic phenomenon.

CI-920 and its analogues are devoid of antimicrobial activity. Thus, it does not fall in the classical definition of an antibiotic. It has excellent antitumor activity, particularly against murine leukemias. The activity appears to be due to the inhibition of DNA biosynthesis; the exact mode of action is currently being studied.

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