PD 116,152, A NOVEL PHENAZINE ANTITUMOR ANTIBIOTIC

DISCOVERY, FERMENTATION, CULTURE CHARACTERIZATION AND BIOLOGICAL ACTIVITY

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(Received for publication October 9, 1985)

A novel phenazine antitumor antibiotic is described, produced by *Streptomyces lomondensis* subsp. *galanosa* NRRL 15738. The antibiotic is selectively active *versus* the bacterium *Streptococcus pneumoniae* (MIC <0.46 μg/ml); the antitumor activity *versus* murine P388 leukemia is T/C 149.

A novel phenazine type compound, PD 116,152[1] (Fig. 1) was discovered in the course of our screening program for antitumor antibiotics. The screening system involved a microorganism particularly sensitive to DNA-active compounds.

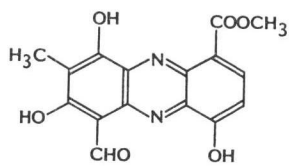
The producing organism has been identified as a new *Streptomyces*. The phenazine antibiotic was found to resemble lomofungin.[2] This paper describes the screening method, taxonomy of the producing-organism, fermentation and biological properties of the compound.

Materials and Methods

Screening System

A mutant organism *Escherichia coli* CM871,[3] characterized by 3-gene deletions (*uvrA*, *recA*, and *lexA*) was obtained from the University of Auckland, New Zealand (c/o Dr. L. FERGUSON). A slow-growing variant of this organism was subsequently isolated, designated as *E. coli* CM871NZ, and was used as the assay organism. A wild type strain, WP2NZ, was used as a control.

Fig. 1. PD 116,152.



The screening system was carried out as a disk test. Thus, the fermentation samples were disked on a plate seeded with the test organism. For control, an agar plate seeded with the wild type strain, *E. coli* WP2NZ was disked with the same fermentation samples.

The agar plating medium included an agar base layer and a soft-agar overlay. The base agar consisted of K_2HPO_4 0.7%, KH_2PO_4 0.3%, sodium citrate 0.094%, $MgSO_4 \cdot 7H_2O$ 0.01%, $(NH_4)_2SO_4$ 0.1%, dextrose 2%, casein hydrolysate 5%, L-tryptophan 0.2% and agar 1.5%. The pH was adjusted to 7.3 before autoclaving. Glucose, casein hydrolysate and L-tryptophan were sterilized separately. The soft-agar overlay consisted of agar 1.0%.

The assay plate was prepared by pouring 30 ml of the molten base agar onto a 150×25 mm (Lab-Tek) Petri plate; after it was solidified, 10 ml of the soft-agar seeded with the organism were evenly overlayed. Paper disks (12.7 mm) dipped in the fermentation beers were laid onto the plate and the plate was incubated overnight at 37°C. Fermentation samples considered positive in the test showed inhibition zones *versus E. coli* CM871NZ at least 4 mm larger than the zones in the control *E. coli* WP2NZ.

Culture Characterization

The culture was isolated from a soil collected in St. Thomas, Virgin Islands. The soil was plated on a modified Lindenbein medium.[4]

Culture characterization was carried out following the ISP procedure.[5] In addition CIM-23 medium[6] was used. Cell wall and whole cell analyses were carried out following the procedure of BECKER *et al.*[7] and LECHEVALIER,[8] respectively.

Fermentation

Stock cultures of the organism were maintained in lyophilized vials, and working cultures stored as cryovials in liquid nitrogen refrigerator (Union Carbide, Indianapolis). To start a fermentation, the contents of a thawed cryovial was used to inoculate a 300-ml seed flask (Bellco, Shallow baffle) containing 50 ml of seed medium. The seed medium consisted of 0.5% yeast hydrolysate (Amberex 1003, Amber Labs.), Cerelose 0.1%, dextrin (Amidex B411, Corn Products) 2.4%, hydrolyzed peptone (N-Z case, Humko-Sheffield) 0.5%, spray-dried meat solubles (Daylin Labs.) 0.3% and $CaCO_3$ 0.2%.

The production of the antibiotic was carried out in 300-ml shake-flasks, 30-liter stirred-jars, or in 760-liter fermentors. The production medium consisted of maltose (Eastern Chem.) 2.5%, distillers solubles (Grain Processing) 0.5%, safflower meal (PVO International) 0.75%, torula yeast (Rhinelander Paper Co.) 0.2%, NaCl 0.1% and $CaCO_3$ 0.25%. The fermentation conditions were as follows: shake-flask, 50 ml/300-ml flask, 200 rpm shaker speed (Model G-53 New Brunswick Co.); 30-liter stirred-jar, 16 liters/jar 1 vol/vol/minute air, 300 rpm; and 760-liter fermentor, 600 liters/tank, 0.75 vol/vol/minute air, 155 rpm. The fermentations were carried out for 72 hours at 33°C.

Assay and Antitumor Activity

The antibiotic was assayed by both inhibition zone *versus E. coli* CM871NZ and by high pressure liquid chromatography (HPLC). The HPLC assay method involved a 4.1 (ID)×250 mm PRP-1 column (Hamilton Co., Reno) and a mobile phase consisting of a linear gradient form of 0.025 M, pH 9.5, borate buffer - acetonitrile - methanol (90 : 5 : 5) at time zero, to 0.025 M, pH 9.5, borate buffer - acetonitrile - methanol (70 : 25 : 5) over a course of seven minutes at a flow rate of 2.0 ml/minute. The retention time of the antibiotics in this system was 3.5 minutes.

The *in vivo* antitumor activity was evaluated *versus* P388 murine lymphocytic leukemia tumor cell line in CDF, mice.[9] The tumor cells were injected intraperitoneally (ip) on day 0, and the antibiotic was administered ip on day 1 through 9.

Antimicrobial Activity

The antimicrobial activity of the compound was evaluated by the broth dilution method.[10] Because of the poor water solubility, the antibiotic was dissolved in methanol then diluted with distilled water to bring the final solvent concentration to 10%. Subsequent dilutions were made in the media previously dispensed in the microdilution trays.

Results

Morphological and Cultural Characteristics

Whole cell and cell wall analyses revealed LL-2,6-diaminopimelic acid (L-DAP) and glycine with no characteristic sugars. The organism produced aerial and substrate mycelia typical of *Streptomyces*.

The growth characteristics of the organism on different media is shown on Table 1, carbon utilization on Table 2, and physiological properties on Table 3. In reviewing the literature, some of these characteristics resembled *Streptomyces lomondensis* NRRL 3252.[11] For this reason side-by-side comparative studies were carried out for the two organisms. Some notable differences include spore chain morphology, spore wall ornamentation (Fig. 2), nitrate reduction, and production of soluble pigments (Table 4).

Fermentation

The fermentation pattern of the organism in a 756-liter fermentor is shown in Table 5. The pro-

Table 1. Growth characteristics of *Streptomyces lomondensis* subsp. *galanosa* NRRL 15738.

Medium	Aerial growth[a]	Mycelium color	Substrate growth[a]	Mycelium color	Diffusable pigments
Yeast extract - malt extract agar	++	19dc Aqua grey	+++	3pl Clove brown	3gc Light tan
Oatmeal agar	+	Slight white	+++	3ne Butterscotch	3ca Pearl pink
Inorganic salts - starch agar	+++	19dc Aqua grey	+++	3pn Coffee	Colorless
Glycerol - asparagine agar	+	19dc Aqua grey	++	4pl Orange rust	Colorless
CIM23	+++	19dc Aqua grey	+++	3pl Clove brown	3gc Light tan

[a] +++ Good, ++ moderate, + poor.

Table 2. Carbohydrate utilization of strain WP4611 *Streptomyces lomondensis* subsp. *galanosa* NRRL 15738.

Test	Results
L-Arabinose	+
D-Fructose	+
D-Galactose	+
D-Glucose	+
i-Inositol	+
Inulin	+
Maltose	+
D-Mannitol	+
Melibiose	+
Raffinose	+
Rhamnose	+
Salicin	−
Sucrose	+
D-Xylose	+

+ Growth, − no growth.

Table 3. Physiological properties of *Streptomyces lomondensis* subsp. *galanosa* NRRL 15738.

Growth after 7 days incubation:	
11°C	−[a]
24°C	+++
28°C	+++
33°C	++
37°C	+++
45°C	−
Nitrate reduction:	Positive in 48 hours
Gelatin liquefaction:	Positive in 6 days-brown pigment
Skim milk peptonization:	Negative ater 21 days
Production of:	
H_2S	Positive
Melanin	Positive
Gram stain	Positive

[a] +++ Good, ++ moderate, + poor, − no growth.

Fig. 2. Electron micrographs of spores of *Streptomyces lomondensis* subsp. *galanosa* NRRL 15738 (A) showing warty ornamentation compared to the spiny spores of *S. lomondensis* NRRL 3252 (B) (×46,100).

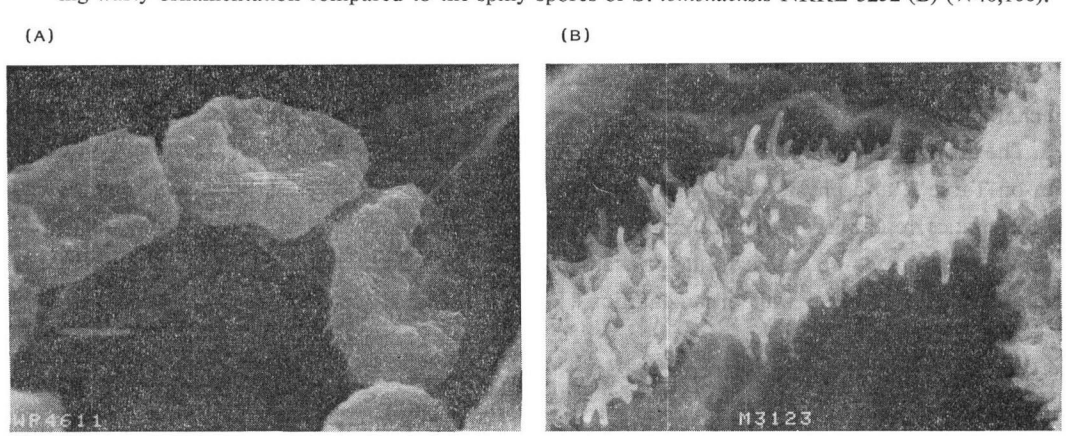


Table 4. Side-by-side comparison of *Streptomyces lomondensis* subsp. *galanosa* NRRL 15738.

Property		Cultural description	
		WP4611	M3123
Whole cell analysis		L-DAP; no characteristic sugar	L-DAP; no characteristic sugar
Spore chain morphology		Long tight spirals few open spirals rare straight chains	Mostly straight and open spirals few tighter spirals - shorter than WP4611.
Spore wall ornamentation		Rough to warty	Spiny
Diffusable pigments:	ISP-1	Brown	None
(melanin formation)	ISP-6	Black	Black
	ISP-7	Brown	Brown
Gelatin liquefaction		Positive-brown	Positive-brown
Nitrate reduction		Positive	Negative
Culture growth		No growth at 11 and 42°C	No growth at 11 and 42°C
Milk coagulation		Negative	Negative
Milk peptonization		Negative (brown pigment)	Negative (light brown pigment)
Aerial mycelia:	ISP-2	Aqua grey (19dc)	Aqua grey (19dc)
	ISP-3	Slight white	Slight white
	ISP-4	Aqua grey (19dc)	Aqua grey (19dc)
	ISP-5	Aqua grey (19dc)	Aqua grey (19dc)
	CIM23	Aqua grey (19dc)	Aqua grey (19dc)
Substratal mycelia:	ISP-2	Clove brown (3pl)	Brown mahogany (6pi)
	ISP-3	Butterscotch (3ne)	Maple (4ng)
	ISP-4	Coffee (3pn)	Dark brown (4pn)
	ISP-5	Orange rust (4pl)	Wine (7pg)
	CIM23	Clove brown (3pl)	Clove brown (3pl)

Table 5. Production of PD 116,152 in a 756-liter fermentor.

Fermentation stage (hours)	Inhibitory zone (mm)*		HPLC assay (μg/ml)
	E. coli WP2NZ	*E. coli* CM871NZ	
24	17.5	24.5	—
36	15.5	23.5	—
48	15.5	22.5	—
60	15.0	22.0	—
72	0	21.0	4.0

* 12.7 mm disks.

duction of the antibiotic started at about the 24-hour stage. In this example the fermentation fluid was harvested at the 72-hour stage with a yield of ~ 4 μg/ml.

Antimicrobial and Antitumor Activities

The antibiotic was notably active *versus Streptococcus pneumoniae* (MIC <0.46 μg/ml) with marginal activity *versus Branhamella catarrhalis*, *Micrococcus luteus* and *S. pyogenes* (Table 6). The compound was inactive *versus* the fungi tested in this study.

The antitumor activity *versus* P388 murine lymphocytic leukemia *in vivo*, expressed as % T/C was 149. Toxicity was observed at 20 mg/kg at the described dosing schedule.

Discussion

The initial fermentation beer was active *versus* the microbial screen but activity was not detected

in the L1210 tissue culture screen. This indicates the feasibility and effectivity of a microbial system as a front line screen for antitumor compounds. A purified PD 116,152, however, showed cytotoxicity *versus* L1210 in tissue culture (ID_{50} 0.17 μg/ml) but still considerably less than CI-920[12] (ID_{50} 0.073 μg/ml) or CI-940[13] (ID_{50} 0.12 ng/ml).

PD 116,152 showed a very narrow antimicrobial spectrum with excellent activity *versus* *S. pneumoniae* and marginal activity *versus* other microorganisms. Other phenazine type antibiotics, lomofungin[2] and 1,6-dihydroxy-2-chlorophenazine[14] have shown potent and broad antimicrobial activities. The main differences of the above antibiotics from PD 116,152 are certain side groups, indicating the amenability of the compound to structural modification and corresponding changes in biological activity.

Table 6. Antimicrobial activity of PD 116,152.

Microorganisms	MIC (μg/ml)
Escherichia coli 04863	>1,000
Salmonella typhimurium TA 1535	1,000
Branhamella catarrhalis 03596	111
Pseudomonas aeruginosa 05111	>1,000
Micrococcus luteus 05064	111
Staphylococcus aureus 02482	>1,000
Streptococcus pyogenes C203	111
S. pneumoniae SV1	<0.46
Enterococcus faecalis 05045	1,000
Bacillus cereus 04810	333
B. megaterium 066	1,000
Saccharomyces cerevisiae S 288C-alpha	>1,000
Schizosaccharomyces pombe M 1388	>1,000
Rhodotorula aurantiaca M 1508	>1,000
Torulopsis albida M 1390	1,000
Mucor parasiticus M 2652	1,000
Rhizopus japonicus M 1577	>1,000

Notable differences were obseved between the PD 116,152 and lomofungin-producing cultures, particularly the spore chain morphology, spore wall ornamentation, melanin formation, nitrate reduction and soluble pigment production. The above differences however, do not seem to be significant enough to warrant the assignment of a new species. Therefore, we regard the PD 116,152-producing organism as a new subspecies for which we propose the name *Streptomyces lomondensis* subsp. *galanosa*. The organism has been deposited with the Northern Research Laboratory (NRRL), Peoria, IL with the accession number NRRL 15738.

Acknowledgment

This work was supported in part by the National Cancer Institute (NCI), USA, contract N01-CM-07379. We thank Dr. J. FRENCH and his staff in the Chemistry section for providing us the structure of PD 116,152, and to the NCI for the P388 *in vivo* data.

References

1) SMITKA, T. A.; R. H. BUNGE, J. H. WILTON, G. C. HOKANSON, J. C. FRENCH & J. CLARDY: PD 116,152, a new antitumor phenazine antibiotic. J. Antibiotics, in preparation.
2) TIPTON, C. D. & K. L. RINEHART: Lomofungin I. Degradative studies of a new phenazine antibiotic. J. Am. Chem. Soc. 92: 1425~1426, 1970
3) TWEATS, D. J.; M. H. L. GREEN & W. J. MURIEL: A differential killing assay for mutagens and carcinogens based on an improved repair-deficient strain of *Escherichia coli*. Carcinogenesis 2: 189~194, 1981
4) WILLIAMS, S. T. & T. CROSS: Actinomycetes. Methods Microbiol. 4: 295~334, 1971
5) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
6) HICKEY, R. J. & H. D. TRESNER: A cobalt-containing medium for the sporulation of *Streptomyces* species. J. Bacteriol. 64: 891~892, 1952
7) BECKER, B.; M. P. LECHEVALIER & H. A. LECHEVALIER: Chemical composition of cell wall preparations for strains of various form-genera of aerobic actinomycetes. Appl. Microbiol. 13: 236~243, 1965
8) LECHEVALIER, M. P.: Identification of aerobic actinomycetes of chemical importance. J. Lab. Clin. Med. 71: 934~944, 1968
9) GOLDIN, A. & J. M. VENDITI: The new NCI screen and its implications for clinical evaluation. Cancer Res. 70: 5~20, 1980
10) WASHINGTON, J. A. & V. L. SUTTER: Dilution susceptibility test: Agar and macro-broth dilution procedures. *In* Manual of Clinical Microbiology. *Ed.*, E. H. LENETTE, pp. 453~458, American Soc. Microbiol., Washington, D.C., 1980

11) BERGY, M. E. & L. E. JOHNSON (Upjohn Co.): Antibiotic lomondomycin and method of production. U.S. Patent 3,359,165, Dec. 19, 1967
12) TUNAC, J. B.; B. D. GRAHAM & W. E. DOBSON: Novel antitumor agents CI-920, PD 113,270 and PD 113,271. I. Taxonomy, fermentation and biological properties. J. Antibiotics 36: 1595~1600, 1983
13) TUNAC, J. B.; B. D. GRAHAM, W. E. DOBSON & M. D. LENZINI: Novel antitumor antibiotics. CI-940 (PD 114,720) and PD 114,721. Taxonomy, fermentation and biological activity. J. Antibiotics 38: 460~465, 1985
14) PATEL, M.; V. HEGDE, A. C. HORAN, V. P. GULLO, D. LOEBENBERG, J. A. MARQUEZ, G. H. MILLER, M. S. PUAR & J. A. WAITZ: A novel phenazine antifungal antibiotic, 1,6-dihydroxy-2-chlorophenazine. Fermentation, isolation, structure and biological properties. J. Antibiotics 37: 943~948, 1984