A novel phenazine antitumor antibiotic is described, produced by *Streptomyces lomondenensis* 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 (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. 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, 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.

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$_2$HPO$_4$, 0.7%, KH$_2$PO$_4$, 0.3%, sodium citrate 0.094%, MgSO$_4$·7H$_2$O 0.01%, (NH$_4$)$_2$SO$_4$ 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. In addition to the ISP procedure, CIM-23 medium was used. Cell wall and whole cell analyses were carried out following the procedure of BECKER et al. and LECHEVALIER, 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 CaCO₃ 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 CaCO₃ 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) x 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. 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. 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. 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.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Aerial growtha</th>
<th>Mycelium color</th>
<th>Substrate growtha</th>
<th>Mycelium color</th>
<th>Diffusible pigments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast extract - malt extract agar</td>
<td>++</td>
<td>19dc</td>
<td>+++</td>
<td>3pl</td>
<td>3gc</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aqua grey</td>
<td></td>
<td>Clove brown</td>
<td>Light tan</td>
</tr>
<tr>
<td>Oatmeal agar</td>
<td>+</td>
<td>Slight white</td>
<td>+++</td>
<td>3ne</td>
<td>3ca</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aqua grey</td>
<td></td>
<td>Butterscotch</td>
<td>Pearl pink</td>
</tr>
<tr>
<td>Inorganic salts - starch agar</td>
<td>+++</td>
<td>19dc</td>
<td>+++</td>
<td>3pn</td>
<td>Colorless</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aqua grey</td>
<td></td>
<td>Coffee</td>
<td></td>
</tr>
<tr>
<td>Glycerol - asparagine agar</td>
<td>+</td>
<td>19dc</td>
<td>++</td>
<td>4pl</td>
<td>Colorless</td>
</tr>
<tr>
<td>CIM23</td>
<td>+++</td>
<td>19dc</td>
<td>+++</td>
<td>3pl</td>
<td>3gc</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aqua grey</td>
<td></td>
<td>Clove brown</td>
<td>Light tan</td>
</tr>
</tbody>
</table>

*a* + + + Good, ++ moderate, + poor.

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

<table>
<thead>
<tr>
<th>Test</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Arabinose</td>
<td>+</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>+</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>+</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>+</td>
</tr>
<tr>
<td>D-Inositol</td>
<td>+</td>
</tr>
<tr>
<td>Inulin</td>
<td>+</td>
</tr>
<tr>
<td>Maltose</td>
<td>+</td>
</tr>
<tr>
<td>L-Mannitol</td>
<td>+</td>
</tr>
<tr>
<td>Melibiose</td>
<td>+</td>
</tr>
<tr>
<td>Raffinose</td>
<td>+</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>+</td>
</tr>
<tr>
<td>Salicin</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>+</td>
</tr>
</tbody>
</table>

+ Growth, − no growth.

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

<table>
<thead>
<tr>
<th>Growth after 7 days incubation:</th>
</tr>
</thead>
<tbody>
<tr>
<td>11°C</td>
</tr>
<tr>
<td>24°C</td>
</tr>
<tr>
<td>28°C</td>
</tr>
<tr>
<td>33°C</td>
</tr>
<tr>
<td>37°C</td>
</tr>
<tr>
<td>45°C</td>
</tr>
</tbody>
</table>

Nitrile reduction: Positive in 48 hours
Gelatin liquefaction: Positive in 6 days-brown pigment
Skim milk peptonization: Negative after 21 days
Production of:

H₂S 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).
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 effectiveness 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 (ID₅₀ 0.17 µg/ml) but still considerably less than CI-920 (ID₅₀ 0.073 µg/ml) or CI-940 (ID₅₀ 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 and 1,6-dihydroxy-2-chlorophenazine 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.

Notable differences were observed 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.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>MIC (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli 04863</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>Salmonella typhimurium TA 1535</td>
<td>1,000</td>
</tr>
<tr>
<td>Branhamella catarrhalis 03956</td>
<td>111</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa 05111</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>Micrococcus luteus 05064</td>
<td>111</td>
</tr>
<tr>
<td>Staphylococcus aureus 02482</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>Streptococcus pyogenes C203</td>
<td>111</td>
</tr>
<tr>
<td>S. pneumoniae SV1</td>
<td>&lt;0.46</td>
</tr>
<tr>
<td>Enterococcus faecalis 05045</td>
<td>1,000</td>
</tr>
<tr>
<td>Bacillus cereus 04810</td>
<td>333</td>
</tr>
<tr>
<td>B. megaterium 066</td>
<td>1,000</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae S 288C-alpha</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>Schizosaccharomyces pombe M 1388</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>Rhodotorula aurantiaca M 1508</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>Torulopsis albida M 1390</td>
<td>1,000</td>
</tr>
<tr>
<td>Mucor parasiticus M 2652</td>
<td>1,000</td>
</tr>
<tr>
<td>Rhizopus japonicus M 1577</td>
<td>&gt;1,000</td>
</tr>
</tbody>
</table>

References


