# HIMASTATIN†, A NEW ANTITUMOR ANTIBIOTIC FROM *STREPTOMYCES HYGROSCOPICUS*

## I. TAXONOMY OF PRODUCING ORGANISM, FERMENTATION AND BIOLOGICAL ACTIVITY

KIN SING LAM, GRACE A. HESLER, JACQUELINE M. MATTEI, STEPHEN W. MAMBER and SALVATORE FORENZA

Bristol-Myers Squibb Company, Pharmaceutical Research and Development Division, 5 Research Parkway, P.O. Box 5100, Wallingford, Connecticut 06492, U.S.A.

KOJI TOMITA

Bristol-Myers Research Institute, Ltd., Tokyo Research Center, 2-9-3 Shimo-meguro, Meguro-ku, Tokyo 153, Japan

(Received for publication November 29, 1989)

Strain C39108-P210-51 (ATCC 53653), an actinomycete isolated from a soil sample collected in India, was found to produce a new antitumor antibiotic, designated himastatin. Taxonomic studies on its morphological, cultural and physiological characteristics identified this producing strain as *Streptomyces hygroscopicus* C39108-P210-51. Himastatin shows antimicrobial activity against Gram-positive bacteria but it is inactive against Gram-negative bacteria. Himastatin prolongs the life span of mice inoculated with P388 leukemia and B16 melanoma cells.

In the course of our continuing search for novel antitumor antibiotics, an actinomycete strain C39108-P210-51 (ATCC 53653) isolated from a soil sample from Himachal Pradesh State, India, was found to produce a new antitumor antibiotic designated himastatin. Himastatin is isolated as a white crystalline solid having a MW of 1,484 and molecular formula of $C_{72}H_{104}N_{14}O_{20}$. Himastatin exhibits *in vivo* antitumor activity against P388 leukemia and B16 melanoma; in addition, himastatin shows activity against Gram-positive bacteria. Taxonomic studies showed that the producing strain belongs to the species of *Streptomyces hygroscopicus*. Production titers of himastatin in shake flask and fermenter cultures are 180 mg/liter and 155 mg/liter, respectively. The present paper describes the taxonomy of the producing organism, fermentation and biological properties. The isolation and physico-chemical characterization of himastatin will be described in the following paper[1].

### Taxonomy of the Producing Strain

Microscopic observations showed both substrate and aerial mycelia were formed, which were long, well-branched and not fragmented into short filaments. Chains of arthrospores were borne on the aerial hyphae. The spore chain and spore morphology had the following characteristics: 1) spiral spore chains with 2 to 8 turns, 2) monopodially branched sporophores, 3) spores, oval or barrel-shaped (0.5 to 0.7 by 0.5 to 1.2 $\mu$m), 4) spore ornamentation, rugose or smooth with rugose being the dominant ornamentation, and 5) clear delineation of single spores is not evident (electron micrographs). Sporangium, motile spore and sclerotium were not observed.

† Himastatin was originally designated BMY-40800 in Eur. Pat. Appl. 329, 109, Aug. 23, 1989.

The carbon utilization pattern of strain ATCC 53653 was determined by the method of SHIRLING and GOTTLIEB[2] excepting the inclusion of a 3-hour starvation period between the harvesting and inoculation steps. Washed vegetative cells were shaken at 250 rpm and 28°C on a rotary shaker in a liquid version of International Streptomyces Project (ISP) medium No. 9 with no carbon source. As summarized in Table 1, positive utilization of rhamnose, fructose and soluble starch, among others, was observed.

The cell-wall content of strain ATCC 53653 was examined according to the methods described by

Table 1. Carbon utilization of strain C39108-P210-51.

| | |
|---|---|
| Best utilization: | L-Rhamnose, soluble starch, D-fructose |
| Moderate utilization: | Glycerol, D-xylose, lactose, D-galactose, D-glucose, sucrose, trehalose, D-mannitol, raffinose, L-arabinose |
| Doubtful utilization: | Inositol, melibiose |
| No utilization: | Maltose, sorbose, dulcitol, salicin, D-mannose, melezitose, D-ribose, D-arabinose, cellobiose |

Table 2. Cultural characteristics of strain C39108-P210-51.

| Medium | Growth of vegetative mycelium | Reverse color | Aerial mycelium | Diffusible pigment |
|---|---|---|---|---|
| Tryptone-yeast extract broth (ISP No. 1) | Moderate, not turbid | — | None | None |
| Yeast extract-malt extract agar (ISP No. 2) | Moderate | Mustard brown, [5; E6] | Black | Brown, [6; E5] |
| Oatmeal agar (ISP No. 3) | Scant | Cream, [4; A3] to gray | Black | None |
| Inorganic salts-starch agar (ISP No. 4) | Scant | Cream, [4; A3] to gray | Black | None |
| Glycerol-asparagine agar (ISP No. 5) | Fair to moderate | Sand, [4; B3] | Scant, gray and white | None |
| Peptone-yeast extract-iron agar (ISP No. 6) | Poor, flat | Butter yellow, [4; A5] | None | None |
| Tyrosine agar (ISP No. 7) | Scant | Cream, [4; A3] | None | Apricot (yellow), [5; B6] |
| CZAPEK's sucrose-nitrate agar | Fair | Ivory to champagne, [4; B(3-4)] | Scant, gray and white | Grayish orange, [5; B3] |
| Glucose-asparagine agar | Fair to moderate | Champagne, [4,B4] | Scant, gray and white | Slight, neutral [4; A3] |
| Skim milk agar | Fair, raised | Cream, [4; A3] | None | None |
| Maltose-Tryptone agar | Moderate, raised | Yellowish white, [4; A2] | None | None |
| Nutrient agar | Fair | Dull yellow, [3; B3] | Scant, gray and white | None |
| Tomato juice agar | Good, raised | Brownish orange, [6; C8] | None | Slight, light brown [6; D8] |
| Casein-starch agar | Scant, ivory, flat | Yellowish white, [4; A2] | None | None |
| Modified BENETT's agar | Moderate, raised | Cream, [4; A3] | None | Light yellow, [4; A4] |

Observation after incubation at 28°C for 2 weeks.

Color names and numbers (in brackets) from "Reinhold Color Atlas", by A. KORNERUP and J. J. WANSCHER, Reinhold Publishing Corporation Forlag, Copenhagen, Denmark, 1961.

Table 3. Physiological characteristics of strain C39108-P210-51.

| | | | |
|---|---|---|---|
| Hydrolysis of: | | NaCl, 1~8% (w/v) | + |
| Gelatin | + | 10% (w/v) | − |
| Soluble starch | + | pH 5.0~11.0 | + |
| Potato starch | − | 4.5 and 12 | − |
| Milk coagulation | − | Temperature (°C): | |
| Milk peptonization | + | Growth range | 18~39 |
| Production of: | | No growth | 15 and 41 |
| Nitrate reductase | − or +[a] | Optimal growth | 30~34 |
| Tyrosinase | − | | |
| Tolerance to: | | | |
| Lysozyme, 0.01% (w/v) | − | | |
| 0.001% (w/v) | − | | |

[a] Negative in CZAPEK's sucrose-nitrate broth and positive in peptone-nitrate broth.

BECKER *et al.*[3], YAMAGUCHI[4], and LECHEVALIER and LECHEVALIER[5]. The whole-cell hydrolysate showed the presence of LL-diaminopimelic acid and ribose. The phospholipids contained phosphatidylethanolamine, phosphatidylglycerol and phosphatidylinositol, hence belonged to the type P-II.

The cultural and physiological characteristics of strain ATCC 53653 are given in Tables 2 and 3, respectively. Strain ATCC 53653 grew well in most descriptive media. The aerial mycelium showed grayish color after sporulation and occurred as blackish moistening patches (hygroscopic change) in ISP medium No. 2, 4 and 7 and BENNETT's agar. The substrate mycelium was colorless, grayish yellow or olive brown. Production of melanoid pigments was negative on peptone-yeast extract-iron and tyrosine agars and in Tryptone-yeast extract broth. The optimal growth range was 30~34°C. There was moderate growth at 18°C and 39°C but no growth at 15°C and 41°C. There was no growth on yeast extract-malt extract agar at pH 4.5 but there was growth at pH 5.0. Growth occurred with the medium containing 8% NaCl but no growth at 10%. Strain ATCC 53653 did not tolerate 0.001% lysozyme in Trypticase soy broth plus 1.5% agar. Nitrate was reduced in peptone-nitrate broth; gelatin liquefaction and hydrolysis of soluble starch also gave positive reactions. In Difco skimmed milk medium there was peptonization without coagulation.

The morphological, cultural and physiological characteristics and cell-wall chemistry of strain ATCC 53653 indicate that the strain is classified as *Streptomyces hygroscopicus* in comparison with the diagnostic characteristics of the type strain (NRRL 2387).[6]

## Fermentation

Strain ATCC 53653 was grown in test tubes on agar slants of yeast extract-malt extract agar supplemented with $CaCO_3$. This medium consisted of glucose 0.4%, yeast extract 0.4%, malt extract 1%, calcium carbonate 0.15% and agar 1.5%. The culture was incubated for 10 days at 28°C. To prepare an inoculum for the production phase, the surface growth from the slant culture was transferred to a 500-ml Erlenmeyer flask containing 100 ml of sterile medium consisting of glycerol 2%, fishmeal 1% and calcium carbonate 0.5%. This vegetative culture was incubated at 28°C for 72 hours on a gyrotary shaker (Model 53, New Brunswick Scientific Co.) set at 250 rpm. Five ml of vegetative culture were transferred to a 500-ml Erlenmeyer flask containing 100 ml of production medium having the same composition as the vegetative medium. The production culture was incubated at 28°C and 250 rpm on the same shaker for 4~5 days. For production in fermenters 1.5 liters of vegetative culture were transferred to 30-liter production medium. The incubation temperature was 28°C, the agitation rate was 250 rpm and the air flow was 1

Table 4. Effect of himastatin on P388 leukemia.

| Treatment schedule | Dose, ip (mg/kg/injection) | Effect MST (% T/C) |
|---|---|---|
| QD 1 to 5 days | 3.2 | 75 |
| | 1.6 | 125 |
| | 0.8 | 140 |
| | 0.4 | 130 |
| | Control | 100 |

Tumor inoculum: $10^6$ ascites, ip.
Host: $CDF_1$ mice.
Evaluation: MST = medium survival time.
Effect: % T/C = (MST treated/MST control) × 100.
Criteria: % T/C ≥ 125 considered significant antitumor activity.

Table 5. Effect of himastatin on B16 melanoma.

| Treatment schedule | Dose, ip (mg/kg/injection) | Effect MST (% T/C) |
|---|---|---|
| QD 1 to 9 days | 2.5 | Toxic |
| | 1.2 | 136 |
| | 0.6 | 136 |
| | 0.3 | 128 |
| | Control | 100 |

Tumor inoculum: 0.5 ml 10% cell homogenate, ip.
Host: $BDF_1$ mice.
Evaluation: MST = medium survival time.
Effect: % T/C = (MST treated/MST control) × 100.
Criteria: % T/C ≥ 125 considered significant antitumor activity.

Table 6. Antimicrobial spectrum of himastatin.

| Test organism | MIC (μg/ml) | Test organism | MIC (μg/ml) |
|---|---|---|---|
| *Entercoccus faecalis* A20688 | 0.5 | *E. coli* A20697 | >500 |
| *E. faecalis* A25707 (ATCC 29212) | 0.5 | *E. coli* A9751 (ATCC 33176) | 2 |
| *E. faecalis* A25708 (ATCC 33186) | 0.25 | *Klebsiella pneumoniae* A9664 | >500 |
| *Staphylococcus aureus* A9537 | 0.5 | *K. pneumoniae* A20468 | >500 |
| *S. aureus* A20698 | 1 | *Proteus vulgaris* A21559 | >500 |
| *S. aureus* A24407 (ATCC 29213) | 1 | *Pseudomonas aeruginosa* A9843 | >500 |
| *Bacillus subtilis* A9506-A (ATCC 6633) | 2 | *P. aeruginosa* A20235 (ATCC 23389) | >500 |
| *Escherichia coli* A15119 | >500 | *P. aeruginosa* A21508 (ATCC 27853) | >500 |

volume per minute. The back pressure of the fermenter was set at 0.35 kg/cm$^2$.

The production of himastatin in the fermentation was monitored by HPLC using a C-18 reversed-phase column (μBondapak, 3.9 × 300 mm, Waters Associates). The solvent system was acetonitrile-tetrahydrofuran-water (4 : 1 : 5) and detector wavelength was set at 292 nm. The fermentation extract was processed by extracting the culture broth with an equal volume of ethyl acetate. Fifty μl of the extract were used for HPLC analysis. The production of himastatin in shake flask and fermenter culture after 120 hours of fermentation averaged 165 ~ 180 mg/liter and 145 ~ 155 mg/liter, respectively.

## Biological Activity

The *in vivo* antitumor activity of himastatin was evaluated against lymphocytic leukemia P388 in $CDF_1$ and B16 melanoma in $BDF_1$ mice according to previously described protocols[7,8]. The results are summarized in Tables 4 and 5. The highest dosage evaluated in P388 leukemia assay (3.2 mg/kg/injection (ip) for five daily injections) caused the death of two of six mice by day 5 and so was considered too toxic a dose level. The effective dose levels ranged from 0.4 to 1.6 mg/kg with the maximum effect (40% increase of life span) achieved at a dose of 0.8 mg/kg on a day 1 to 5 treatment schedule. Prolongation of survival of mice inoculated with B16 melanoma was observed at dose levels ranging from 0.3 to 1.2 mg/kg on a day 1 to 9 treatment schedule.

The antimicrobial spectrum of himastatin was determined by serial broth dilution method using nutrient broth (Difco). The results are summarized in Table 6. Himastatin was effective against Gram-positive bacteria but inactive against Gram-negative bacteria tested.

## Conclusion

Culture C39108-P210-51, identified as a species of *S. hygroscopicus*, was found to produce a new antitumor antibiotic. This compound, designated himastatin, possesses *in vivo* activity against P388 leukemia in $CDF_1$ mice, B16 melanoma in $BDF_1$ mice, and *in vitro* activity against Gram-positive bacteria. The isolation and structural determination of himastatin was facilitated by the adequate production of the antibiotic (155 mg/liter) in fermenter by strain C39108-P210-51. The isolation and physico-chemical characterization will be described in the following paper.

Acknowledgments

The authors wish to acknowledge the assistance of Mr. KENNETH DEN BLEYKER for the determination of the antimicrobial spectrum of himastatin, and Mr. JOHN L. CLARK, Ms. SUSAN M. HONYOTSKI and Ms. CHRISTINE E. ROBINSON for performing the *in vivo* antitumor assays.

## References

1) LEET, J. E.; D. R. SCHROEDER, B. S. KRISHNAN & J. A. MATSON: Himastatin, a new antitumor antibiotic from *Streptomyces hygroscopicus*. II. Isolation and characterization. J. Antibiotics 43: 961~966, 1990
2) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
3) BECKER, B.; M. P. LECHEVALIER & H. A. LECHEVALIER: Chemical composition of cell-wall preparations from strains of various form-genera of aerobic actinomycetes. Appl. Microbiol. 13: 236~243, 1965
4) YAMAGUCHI, T.: Comparison of the cell-wall composition of morphologically distinct actinomycetes. J. Bacteriol. 89: 444~453, 1965
5) LECHEVALIER, M. P.& H. A. LECHEVALIER: Chemical methods as criteria for the separation of nocardiae from other actinomycetes. Biology of the Actinomycetes and related organisms. 11: 78~92, 1976
6) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type strains of *Streptomyces*. Int. J. Syst. Bacteriol. 22: 265~394, 1972
7) GERAN, R. I.; N. H. GREENBERG, M. M. MACDONALD, A. M. SCHUMACHER & B. J. ABBOTT: Protocols for screening chemical agents and natural products against tumors and other biological systems (3rd Ed.). Cancer Chemother. Rep. (Part 3) 3: 1~103, 1972
8) BRADNER, W. T.: Transplanted animal tumors. *In* Cancer and Chemotherapy. Vol. 1. *Eds.*, S. T. CROOKE & A. W. PRESTAYKO, pp. 221~227, Academic Press, 1980