LEPTOLSTATIN FROM *Streptomyces* sp. SAM1595, A NEW GAP PHASE-SPECIFIC INHIBITOR OF THE MAMMALIAN CELL CYCLE

I. SCREENING, TAXONOMY, PURIFICATION AND BIOLOGICAL ACTIVITIES

KEIICHI ABE†, MINORU YOSHIDA*, SUEHARU HORINOUCHI and TERUHIKO BEPPU

Department of Agricultural Chemistry, Faculty of Agriculture, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

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Leptolstatin, a new inhibitor of the progression of G1 and G2 phases of the mammalian cell cycle, was discovered through a unique screening system, in which effects of microbial metabolites on the cell cycle progression of the cultured rat fibroblasts were monitored by flow cytometry. The new inhibitor was extracted from the fermentation broth of *Streptomyces* sp. SAM1595 with ethyl acetate, and purified by silica gel column chromatography and HPLC. Leptolstatin showed a strong cytostatic effect on rat normal fibroblasts 3Y1 with an IC_{50} value of 0.4 ng/ml, but its antimicrobial activity was very weak. A 24-hour treatment of the fibroblast cells with 10 ng/ml of leptolstatin caused an arrest at G1 or G2 phase, as determined by flow cytometry. When the G2-arrested cells were freed from leptolstatin, those containing 4C DNA entered a new S phase without intervening M phase, resulting in the formation of proliferative tetraploid cells.

The eukaryotic cell cycle consists of two major events, DNA replication in S phase and mitotic cell division in M phase, which are separated by two intervening (gap) phases, G1 and G2. Most of the antitumor agents are inhibitors of DNA metabolism or mitotic apparatus, and very few agents are known as the inhibitors specific for G1 and G2 phase. However, recent extensive studies have revealed that complicated signal transduction systems including many proto-oncogene functions are working in these gap phases. Various steps of such signal transduction pathway are activated or short-circuited by oncogenes, thereby growth is less restricted in cancer cells[1]. Therefore it seems reasonable that the regulatory events occurring in the gap phases are more suitable targets for selective antitumor agents.

Trichostatin A[2], which was rediscovered by our group to be a potent inducer of Friend leukemia cell differentiation, was found to arrest the mammalian cell cycle at G1 and G2 phases. We also discovered leptomycin B[3] as another member of the gap phase-specific cell cycle inhibitors. These agents showed potent cytotoxic effects on the virus-transformed rat fibroblast cells, whereas their effects on the normal cells were static and reversible. The selectivity of these agents to the transformed cells suggested the possibility that gap phase-specific cell cycle inhibitors could become a new class of potent antitumor drugs with low toxicities. We therefore started a new screening program using a cultured rat fibroblast cell line for the assay and analyzing the effect on the cell cycle through measurement of cellular DNA contents by flow cytometry. During this screening, we recently found that staurosporine, a potent inhibitor of protein

† Present address: Suntory Institute for Biomedical Research, 120-1, Takahama, Shimamoto-cho, Mishima-gun, Osaka 618, Japan.

kinases, blocked the cell cycle at both G1 and G2 phases[4].

Here we report on an active substance produced by a *Streptomyces* strain, which causes G1- and G2-arrest in the cell cycle of normal rat fibroblasts. The active substance was named leptolstatin because of its structural similarity to leptomycins. In this paper, we describe taxonomy of the producing strain, fermentation, isolation, and biological properties of leptolstatin. Physical and chemical properties and structural elucidation of leptolstatin are reported in the accompanying paper[5].

Materials and Methods

Screening

Microbial strains were isolated on agar media from samples of soil, mud and water, which had been collected from various districts in Japan. The media we used were Maltose-Bennett (maltose 1.0%, Polypepton (Difco) 0.2%, meat extract 0.1%, yeast extract (Difco) 0.1%, and agar 2.0%, pH 7.2) and YS medium (yeast extract 0.2%, soluble starch 1.0% and agar 2.0%, pH 7.0). The actinomycetes thus isolated were inoculated in a 21-mm test tube containing 10 ml of YN medium (soluble starch 2.0%, glycerol 1.0%, Polypepton 0.5%, malt extract 0.2%, yeast extract 0.5%, NaCl 0.2%, K_2HPO_4 0.05% and $MgSO_4 \cdot 7H_2O$ 0.05%, pH 7.2), and cultured aerobically at 28°C for 5 days with shaking at 300 rpm. An equal volume of acetone was added to the culture broth and the mixture was kept standing for one day at room temperature to extract mycelial contents. Rat fibroblast 3Y1-B cells[6] (referred to as 3Y1) were maintained at 37°C in EAGLE's minimal essential medium supplemented with 12% fetal calf serum in a humidified atmosphere of 5% CO_2. The growing 3Y1 cells were introduced into G0 phase by deprivation of serum for 36 hours and their proliferation was reinitiated synchronously by the addition of 12% serum. The G0-starting synchronous culture obtained in this way was then arrested at early S phase by the treatment with 1 mM hydroxyurea at 10 hours after serum stimulation. After an additional incubation for 14 hours, hydroxyurea was removed to reinitiate the cell cycle synchronously. The early S phase synchronous cultures were treated with the extracts of the isolated microorganisms at a final concentration of 0.1% or 1% and then further cultivated for 24 hours. The cells were collected by centrifugation after trypsinization, and the cell pellet was treated with phosphate-buffered saline (PBS) containing 0.1% NP-40 for isolation of nuclei[7]. DNA contents per nucleus in the cell population were measured by flow cytometry, as described below.

Taxonomy

The producing strain, SAM1595, was characterized taxonomically according to the method of SHIRLING and GOTTLIEB[8]. Color names were used according to the Methuen Handbook of Colour (third edition). Physiological properties and utilization of carbon sources were determined by the method of PRIDHAM and GOTTLIEB[9]. The type of diaminopimelic acid in the cell wall was analyzed by the method of BECKER *et al.*[10].

Fermentation

The stock culture of the producing organism, SAM1595, was inoculated into five 500-ml Sakaguchi flasks, each containing 100 ml of YN medium and cultivated for 5 days at 28°C on a reciprocal shaker (150 rpm). The seed culture (5 × 100 ml) was transferred to a 50-liter jar fermentor containing 30 liters of YN medium, and was cultivated for 60 hours at 28°C with agitation of 300 rpm and with an aeration rate of 24 liters/minute. The culture was then transferred to a 500-liter jar fermentor containing 300 liters of the same medium, and was cultivated for 96 hours at 28°C with agitation of 150 rpm and with an aeration rate of 150 liters/minute.

Antimicrobial Activity

Antimicrobial activity of leptolstatin was determined by the serial agar dilution method using nutrient agar medium for bacteria and Potato Dextrose agar medium for fungi and yeasts. Growth inhibition was observed after 24 hours at 37°C for bacteria and 72 hours at 27°C for fungi and yeasts.

Cytotoxicity and Flow Cytometry

After 3Y1 cells (5×10^4 cells/35 mm dish) had been treated with various concentrations of the agent for 3 days, the cells were washed twice with PBS and then trypsinized. The cell number in each dish was counted with a hemocytometer. The concentrations that inhibits 50% of control growth (IC_{50} values) were calculated to assess the potency of the inhibitory effect.

The distribution of DNA content in individual naked nuclei of 3Y1 cells was determined after 24 hours of cultivation with the extracts of the culture broth or purified compounds. Nuclei were stained with 50 μg/ml propidium iodide (Sigma) and applied to a flow cytometer (Epics C, Coulter) equipped with a 5-W argon ion laser operated at 200 mW at a wavelength of 488 nm.

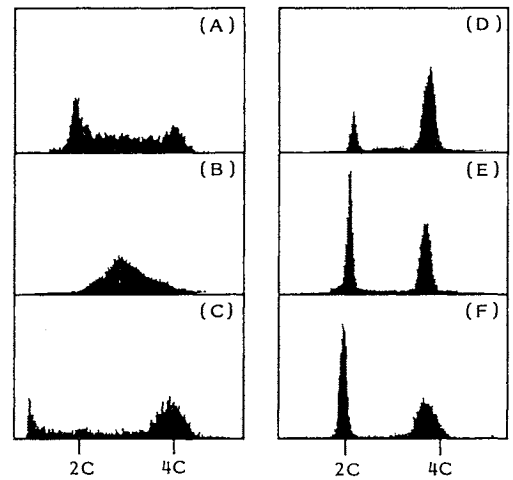
Results

Screening

We devised a screening system for detecting the activity of inhibitors of the cell cycle progression at G1 or G2 phase, in which effects of microbial metabolites on the early S phase synchronous culture of rat 3Y1 fibroblasts were monitored by flow cytometry. Fig. 1A shows a typical profile of DNA content distribution obtained with a control culture 24 hours after hydroxyurea removal; there are two peaks representing 2C DNA (cells in G1 phase) and 4C DNA (G2 and M phases), as well as a broad peak (S phase) between the two peaks. The use of the early S synchronous culture enabled us to discriminate DNA-attacking agents such as mitomycin C from agents inhibiting the progression of the other phases in the cell cycle, since the DNA-attacking agents inhibited the initial progression of the S phase and showed a broad peak between 2C and 4C DNAs (Fig. 1B). It was difficult to discriminate between cells arrested in G2 and M by general flow cytometric analysis, since both of them possess the same DNA content of 4C. However, the use of isolated nuclei instead of fixed cells facilitated the discrimination between them. For example, nuclei of the cells arrested in M phase by vinblastine showed a characteristic pattern with a broad peak of DNA contents lower than 2C in addition to a 4C DNA peak, probably due to individual metaphase chromosomes without their nuclear envelope (Fig. 1C). This pattern was distinctly different from that of the G2 arrest by trichostatin A showing a major sharp peak at 4C (Fig. 1D). Thus, we assumed that the agents inhibiting the progression of G1 or G2 phase could be detected effectively by this procedure.

During the course of our screening program, we found that a strain, named SAM1595, produced an active substance(s) inducing both G1 and G2 arrest in the cell cycle of 3Y1 fibroblasts (Fig. 1F). The DNA distribution pattern of the cells treated with the extract of strain SAM1595 was similar to that with leptomycin B (Fig. 1E), which was reported to cause a complete G1 arrest and a somewhat leaky

Fig. 1. Effects of various growth inhibitors on the progression of mammalian cell cycle.



DNA distribution pattern of untreated control 3Y1 cells (A), treated with 10 ng/ml of mitomycin C (B), 100 ng/ml of vinblastine (C), 100 ng/ml of trichostatin A (D), 10 ng/ml of leptomycin B (E) and 0.1% of the extract of culture broth of the strain SAM1595 (F).

G2 arrest[3]. The absence of round mitotic cells in the culture treated with the extract of strain SAM1595 was confirmed under a light microscope. These results indicate that this strain produces an active substance(s) which inhibits the progression of the cell cycle of 3Y1 fibroblasts in both G1 and G2 phases.

Taxonomy

Microscopic studies of strain SAM1595 showed that aerial mycelia were formed from branched substrate mycelia grown on various agar media. Most of the spores were oval and possessed a hairy surface (Fig. 2). Typical culture characteristics and physiological properties of strain SAM1595 are summarized in Tables 1 and 2. Strain SAM1595 utilized D-glucose, D-fructose, inositol, L-rhamnose, and raffinose, but did not utilize L-arabinose, D-xylose, sucrose, or D-mannitol. The cell wall analysis showed the presence of L,L-diaminopimelic acid. Thus, we concluded that the strain belonged to the genus *Streptomyces*. These characteristics of strain SAM1595 were not identical, but similar, to those reported for *Streptomyces karnatakensis*[11] and *Streptomyces pactum*[12].

Isolation of the Active Substance

A cultured broth (300 liters) of strain SAM1595 was filtered with filter press, and the active substance was extracted from the mycelium with a solvent mixture composed of methanol (30 liters) and ethyl acetate (36 liters). After solvent evaporation, the residue was suspended in water (6 liters) and washed with *n*-hexane (6 liters). The aqueous layer was then extracted three times with ethyl acetate. The extract concentrated *in vacuo* was dissolved in chloroform - methanol (95 : 5) and subjected to silica gel column chromatography (Silica gel 60, 50 × 300 mm) with a gradient of chloroform - methanol from 100 : 0 to 50 : 50 for elution.

Table 1. Cultural properties of strain SAM1595.

Medium	Aerial mycelium	Reverse side color	Soluble pigment
Sucrose - nitrate agar	Thin, grey	Grey	None
Glucose - asparagine agar	Scant, grey	Yellow	None
Glycerol - asparagine agar (ISP)	None	Orange yellow	None
Inorganic salts - starch agar (ISP)	Moderate, grey	Greyish yellow	None
Tyrosine agar (ISP)	Moderate, grey	Greyish yellow	None
Nutrient agar	None	Pale yellow	None
Yeast extract - malt extract agar (ISP)	Thin, grey	Orange yellow	None
Oatmeal agar (ISP)	Thin, grey	Greyish yellow	None

Fig. 2. Scanning electron microscopy of spore chains of strain SAM1595.

Bar indicates 5 μm.

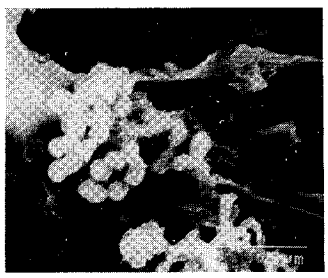


Table 2. Physiological characteristics of strain SAM 1595.

Nitrate reduction	−
Liquefaction of gelatin	+
Starch hydrolysis	+
Coagulation of milk	−
Peptonization of milk	−
Melanin formation	−
Tyrosinase reaction	−
Production of H_2S	−

The active substance was eluted with chloroform-methanol (approx 90:10). The major active fractions were collected and concentrated, and the active substance was further purified by semi-preparative HPLC with an Aquasil silica gel column (Senshu Chemical Co., Ltd., 20×300 mm) using a solvent system composed of chloroform and methanol (98:2, v/v). Finally, the active fractions were applied onto the same column with a solvent system of hexane-ethyl acetate (1:3). This procedure yielded 280 mg of the pure substance.

Biological Activities

The antimicrobial activity of leptolstatin was determined by the agar dilution method. The agent was active only against *Schizosaccharomyces pombe*, but not against all the other microorganisms tested, including Gram-negative and -positive bacteria, fungi and yeasts (Table 3).

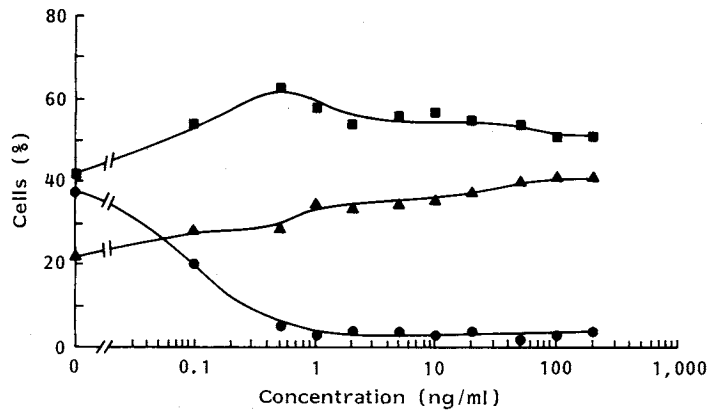
Despite its weak antibiotic activity, leptolstatin showed a strong inhibitory effect on mammalian cells at very low concentrations. The IC_{50} value for rat normal fibroblast 3Y1 cells was determined to be 0.4 ng/ml on the basis of the total cell number after treatment for 3 days. The effect of leptolstatin on the distribution of cellular DNA contents in exponentially growing 3Y1 cells was analyzed by flow cytometry after treatment for 24 hours. Accumulation of the cells with 2C and 4C DNA contents was observed in a wide concentration range of leptolstatin from 1 to 200 ng/ml (Fig. 3), suggesting that leptolstatin had no activity to inhibit DNA synthesis or mitosis even at a high concentration.

The growth inhibition of 3Y1 cells by

Table 3. Antimicrobial activity of leptolstatin.

Test organisms	MIC (μg/ml)
Staphylococcus aureus IAM 1098	>64
S. aureus ATCC 9144	>64
Bacillus subtilis DSM 10	>64
Escherichia coli IAM 1239	>64
E. coli IAM 1264	>64
Pseudomonas aeruginosa IAM 1514	>64
Saccharomyces cerevisiae IAM 4021	>64
Candida albicans IAM 4888	>64
Schizosaccharomyces pombe IAM 4863	4
Penicillium citrinum IAM 7003	>64
Aspergillus niger IAM 2020	>64
Rhizopus oryzae IAM 6006	>64
R. delmar IAM 6038	>64
Mucor javanicus IAM 6087	>64
M. oruxianus ATCC 8099	>64

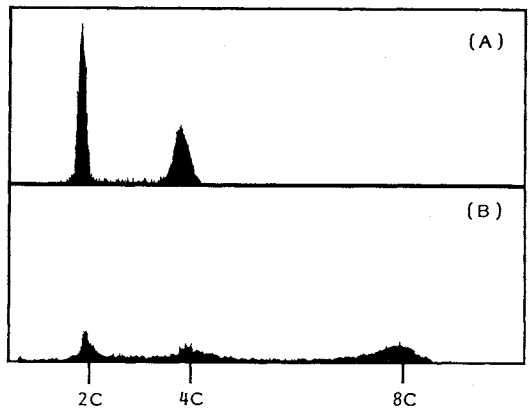
Fig. 3. A dose effect of leptolstatin on the DNA content distribution of exponentially growing 3Y1 cells.
■ G1, ▲ G2/M, ● S.



Each cell population was determined by quantitative flow cytometry after treatment for 24 hours.

leptolstatin was reversible. Interestingly, removal of leptolstatin from the arrested culture induced an extra round of DNA synthesis in G2-arrested cells and produced the cells with DNA contents higher than 4C (Fig. 4). This finding suggests that the diploid G2 cells are converted to tetraploid G1 cells during the G2 arrest and the removal of the agent induced the formation of proliferative tetraploid cells.

Fig. 4. Tetraploid cell formation after leptolstatin treatment.



(A) DNA distribution pattern of the cells treated with 10 ng/ml of leptolstatin for 24 hours. The treated cells were arrested in both G1 and G2 phases.
(B) Appearance of tetraploid cells with DNA contents from 4C to 8C. Leptolstatin was removed from the treated culture (A) and cells cultivated for additional 24 hours in the complete medium.

Discussion

Leptolstatin was discovered as an inhibitor able to block the cell cycle at both G1 and G2 phases through our unique screening system in which the effects of microbial metabolites on the progression of the cell cycle of rat fibroblast cells were directly monitored by flow cytometry. In this screening system, the use of isolated nuclei and the early S phase synchronous culture allowed to exclude DNA-attacking agents and inhibitors of mitotic apparatus.

The present study shows that leptolstatin is a new member of the gap phase-specific inhibitors, in addition to trichostatin A[2], leptomycin B[3] and staurosporine[4], all of them reversibly block the cell cycle of 3Y1 fibroblasts at G1 and G2 phases. Recently the target molecule of trichostatin A was determined to be the nuclear histone deacetylase[13]. Staurosporine has already been reported as a potent inhibitor of protein kinases[14], while the mode of action of leptomycin B is still unknown. The close similarity of chemical structure and biological activities between leptolstatin and leptomycin B suggests that their target molecules are common or related.

The most characteristic activity of trichostatin A and leptomycin B was the ability to induce proliferative tetraploid cell formation after release from the G2 arrest. A similar phenomenon was also observed after the cells had been freed from the leptolstatin arrest. These results suggest that the diploid G2 phase cells can be directly converted to tetraploid G1 phase cells during the long G2 arrest induced by inhibitors irrespective of their target molecules. We assume that the G2-arrested cells gradually lose their "G2 memory" during arrest, probably due to degradation of some factors essential for mitosis. Recent analysis of several mutants of *Schizosaccharomyces pombe* diploidized by a transient heat treatment suggested that one of the factors responsible for such a "G2 memory" was *cdc2* protein kinase[15].

The effect of leptomycin B on the normal fibroblast cells was reversible and static, whereas that on the DNA virus-transformed cells was highly toxic. Moreover, leptomycin B showed *in vivo* antitumor activity against some of experimental tumor[16]. The similarity of leptolstatin to leptomycin B suggests that leptolstatin could also show antitumor effects in some *in vivo* models. Further investigations on potential pharmacological activities against experimental tumors are in progress.

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