Studies on New Antitumor Antibiotics, Leptofuranins A, B, C and D

I. Taxonomy, Fermentation, Isolation and Biological Activities

YOICHI HAYAKAWA, KIN-YA SOHDA, KEIKO FURIHATA, TOMOHISA KUZUYAMA, KAZUO SHIN-YA and HARUO SETO

Institute of Molecular and Cellular Biosciences, The University of Tokyo, Bunkyo-ku, Tokyo 113, Japan

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The retinoblastoma protein (pRB) is inactivated in a wide variety of human cancers. In the course of our screening for antitumor antibiotics by using pRB-inactivated cells, an actinomycete identified as *Streptomyces tanashiensis* was found to produce four new active substances, leptofuranins A, B, C and D. The leptofuranins arrested the growth of normal cells and induced apoptotic cell death against tumor cells and cells transformed with the adenovirus E1A gene.

The retinoblastoma tumor suppressor protein (pRB) plays a central role in mammalian cell cycle control and is inactivated during the development of a wide variety of human cancers[1]. Recently, pRB has been implicated in the regulation of apoptosis in certain cell types[2]. The adenovirus E1A gene products inactivate pRB to stimulate host cell DNA synthesis. However, this function of E1A induces the host cells to undergo apoptosis[3]. For this reason, adenoviruses encode a second function in the E1B gene that prolongs host cell viability by inhibiting apoptosis[4].

In order to search for antitumor substances with selective cytotoxicity against tumor cells, we established pRB-inactivated cell lines by transformation with E1A gene. Primary rat glia cells were transfected with plasmids containing E1A or both E1A and E1B genes[3] to establish immortalized cell lines, RG-E1A-7 and RG-E1-4.

In the course of our screening for antitumor antibiotics by using these transformed cells, an actinomycete identified as *Streptomyces tanashiensis* was found to produce four active substances, leptofuranins A, B, C and D (Fig. 1), which were determined to be new members of the leptomycin family[5] by NMR spectral analysis. The leptofuranins induced apoptotic cell death against pRB-inactivated cells and arrested the growth of normal cells. This paper describes the fermentation, isolation and biological activities of leptofuranins A to D. The physicochemical properties and structure elucidation of the leptofuranins are described in the accompanying paper[6].

Materials and Methods

Microorganism

The producing organism designated 3007-H1 was isolated from a soil sample collected at Shiobara-cho, Nasu-gun, Tochigi Prefecture, Japan.

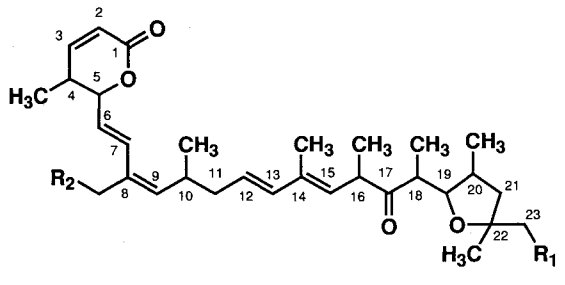
Taxonomic Studies

The characterization and identification of the culture were carried out by the method of the International Streptomyces Project (ISP)[8,9]. For the evaluation of cultural characteristics, the strain was incubated at 27°C for 21 days. Color codes were assigned to the aerial and substrate mass pigments according to the Color Harmony Manual, 3rd Ed., 1950 (Container Corporation of America, Chicago). Cell wall composition was analyzed by the methods of BECKER *et al.*[10].

Cells and Cell Culture

Normal rat glia cells and glia-derived cell lines were maintained in DULBECCO's modified EAGLE's medium supplemented with 10% heat-inactivated fetal calf serum

Fig. 1. Structures of leptofuranins A, B, C and D.



Leptofuranin A	**$R_1 = CH_2OH$**	**$R_2 = H$**
Leptofuranin B	**$R_1 = CH_2OH$**	**$R_2 = CH_3$**
Leptofuranin C	**$R_1 = CHO$**	**$R_2 = H$**
Leptofuranin D	**$R_1 = CHO$**	**$R_2 = CH_3$**

and 0.1% glucose. Other cell lines were cultured in DULBECCO's modified EAGLE's medium supplemented with 10% heat-inactivated fetal calf serum. These cells were grown at 37°C in a humidified atmosphere of 5% CO_2. Rat glia cells were obtained from primary cultures of Wistar rat (18-day embryo) cerebral cortex cells.

DNA Transfection

The adenovirus type 12 E1 gene (E1A+E1B) and an expression vector containing a neomycin-resistant gene, pSV2*neo*, were obtained from the Japanese Cancer Research Resources Bank. The *Bam*HI-*Acc*I fragment of the E1A gene and the *Bam*HI-*Eco*RI fragment of the E1 gene were separately inserted into pSV2*neo* at the *Bam*HI-*Eco*RI site to produce pSVneo-E1A and pSVneo-E1, respectively. Primary rat glia cells were transfected with pSVneo-E1A or pSVneo-E1 at 2.5 μg DNA per 10^5 cells by the calcium phosphate method[7]. One day after transfection, the transfected cultures were replaced at a split ratio of 1:20 and maintained for 3 weeks with refeeding with a medium containing 400 μg/ml of G418, a neomycin analogue. Twelve weeks after transfection, immortalized cells were cloned by limiting dilution and established as RG-E1A-7 and RG-E1-4 cell lines.

MTT Assay

Cells at 50% confluence were plated at one tenth lower cell density and incubated for 3 days with various concentrations of samples. The growth was measured at 570 nm with formazan formation after treatment of the cells with 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 hours at 37°C.

Apoptosis Assays

HeLa cells or WI-38 cells were treated with 100 ng/ml of leptofuranin D for 3 days, and adherent and non-adherent cells were pooled. Chromatin structure was visualized by fluorescence microscopy after staining fixed cells (1×10^6 cells) with Hoechst Dye 33258. For the DNA fragmentation assay, cells (2×10^6 cells) were suspended in 100 μl buffer containing 50 mM Tris-HCl, 10 mM EDTA, 0.5% sodium *N*-lauroylsarcosinate and 100 μg/ml RNase (pH 8.0), and incubated at 37°C for 30 minutes. After treatment with Proteinase K (100 μg/ml) at 55°C for 1 hour, the solution was extracted with phenol and chloroform. The low-molecular-weight DNA was recovered by ethanol precipitation. The samples were suspended in 100 μl of 50 mM Tris-EDTA buffer (pH 8.0) and analyzed by electrophoresis on 2% agarose gels.

Results

Taxonomy

The aerial mycelium of culture 3007-H1 irregularly branched on the main stem and terminated in straight or hooks, forming spore chains with 10~50 spores per chain. The spores were cylindrical to oval in shape (0.4~0.5×1.0~1.3 μm) with a smooth surface. Whole-cell analysis showed that the strain contained L,L-diaminopimelic acid, suggesting cell-wall type I.

The cultural and physiological properties of strain 3007-H1 are summarized in Tables 1 and 2, respectively. On the basis of these characteristics, strain 3007-H1 seems to belong to the genus *Streptomyces*. Among the species of *Streptomyces* described in SHIRLING's reports[11~14], these properties resemble those of *Streptomyces tanashiensis*. A comparison of strain 3007-H1 with *S. tanashiensis*[12] as shown in Table 2 gave a good agreement except for carbon utilization, and therefore, culture 3007-H1 was identified as a strain of *Streptomyces tanashiensis*.

Fermentation

The seed medium consisted of soluble starch 1.0%, molasses 1.0%, meat extract 1.0% and Polypepton 1.0% (pH 7.2). Seed tubes containing 15 ml of the medium were inoculated with a stock culture of the producing strain maintained on a BENNET's agar slant and were incubated on a reciprocal shaker at 27°C for 2 days. The seed culture at 2% was transferred to 500-ml Erlenmeyer flasks containing 100 ml of the seed medium. The flasks were incubated on a rotary shaker at 27°C for 3 days. A 600-ml portion of the culture was inoculated into a 50-liter jar fermenter containing 30 liters of a production medium consisting of glycerol 2.0%, molasses 1.0%, Polypepton 0.6% and calcium carbonate 0.4% (pH 7.2). The fermentation was carried out at 27°C for 4 days under agitation of 300 rpm and aeration of 30 liters/minute.

Isolation

A mycelial cake obtained from the fermentation broth (60 liters) was extracted with 15 liters of acetone. The extract was concentrated and then extracted twice with one liter of ethyl acetate. A chloroform solution (100 ml) of the extract was precipitated by addition of 10 volumes of hexane. The supernatant was applied to a silica gel column (600 ml), which was eluted with hexane-ethyl acetate (1:1) to give two active fractions.

The first fraction was chromatographed on a silica gel column (40 ml) with hexane-ethyl acetate (2:1), and further purified on a silica gel column (40 ml) with chloroform-methanol-29% ammonia water (200:20:1). The active eluate was subjected to reverse-phase HPLC (PEGASIL-ODS, Senshu Scientific Co.) with 70% acetonitrile at 75°C. Two active fractions thus obtained

Table 1. Cultural properties of strain 3007-H1.

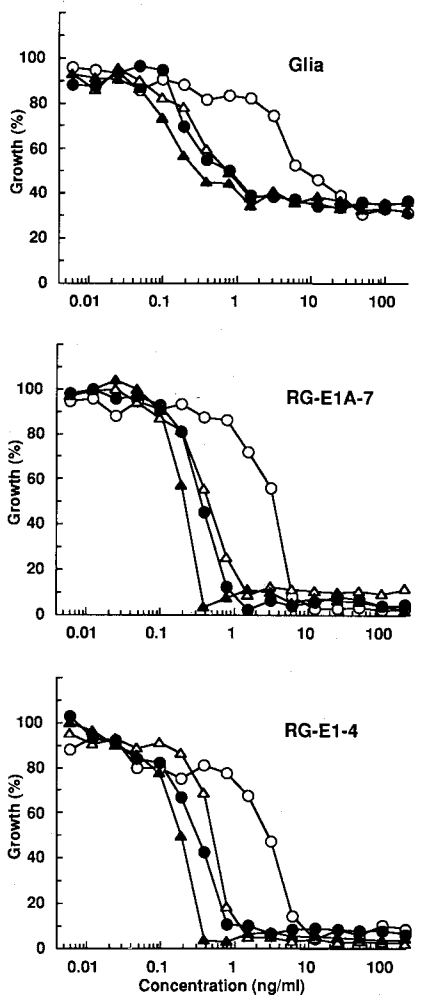
Agar medium	Aerial mycelium	Substrate mycelium	Soluble pigment
Sucrose-nitrate	Gray-color series (e)	Pale yellowish brown (2dc)	None
Glucose-asparagine	Gray-color series (2fe-3fe)	Pale yellowish brown (3le-3lg)	None
Glycerol-asparagine	Gray-color series (d-e)	Brownish gray (2ig-2ni)	None
Inorganic salts-starch	Gray-color series (3fe-5fe)	Light brownish gray (3ec-3lg)	None
Tyrosine	Gray-color series (2ge-3ge)	Brownish gray (4ig-4li)	Light brownish gray (3ge)
Nutrient	No aerial mycelium	Light brownish gray (3ec)	None
Yeast-malt	Gray-color series (2fe-3fe)	Yellowish brown (3ng-3ni)	None
Oatmeal	Gray-color series (2fe-3fe)	Yellowish brown (2gc-2ng)	None

Table 2. Comparison of strain 3007-H1 with *Streptomyces tanashiensis*.

	3007-H1	*Streptomyces tanashiensis*[1,2]
Spore chain morphology		
Straight	+	+
Hooks	+	+
Spore surface		
Smooth	+	+
Color of colony		
Gray	+	+
Reverse color		
Distinctive	−	−
pH sensitivity	−	−
Soluble pigment	−	−
Melanoid pigment	+	+
Hydrolysis of starch	+	+
Coagulation of milk	+	+
Peptonization of milk	±	±
Reduction of nitrate	−	−
Antifungal activity	+	+
Growth at		
10°C	+	±
37°C	−	±
45°C	−	−
Utilization of carbon source		
D-Glucose	+	+
L-Arabinose	−	+
D-Xylose	−	+
D-Fructose	+	±
Sucrose	−	−
L-Rhamnose	−	−
Raffinose	−	−
i-Inositol	−	−
D-Mannitol	−	−
D-Galactose	+	+
Salicin	+	+

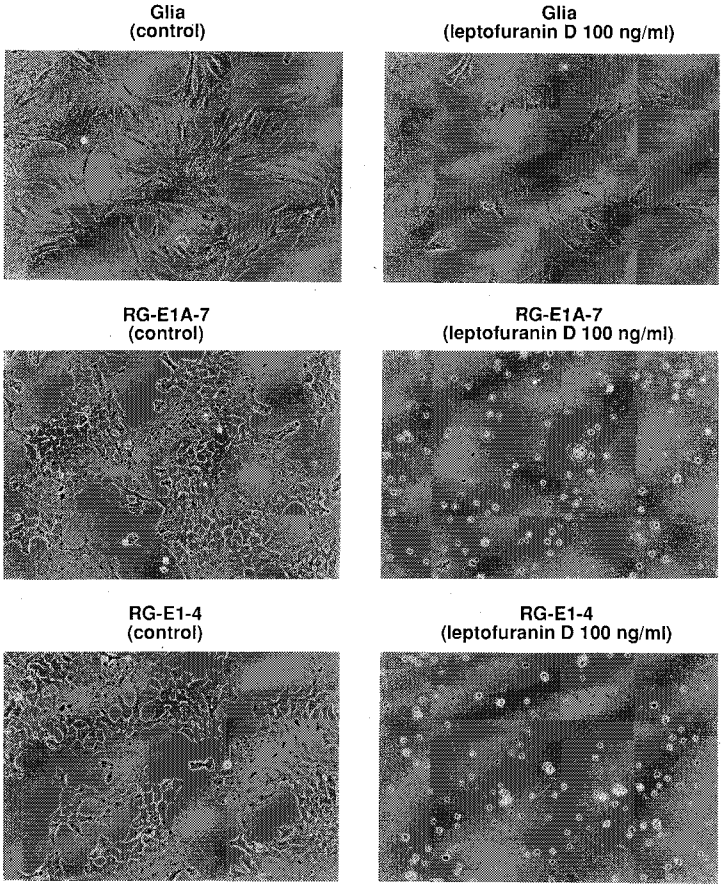
Fig. 2. Effect of leptofuranins A to D on the growth of normal and transformed rat glia cells.

○ Leptofuranin A, △ leptofuranin B, ● leptofuranin C, ▲ leptofuranin D.



Cells were cultured for 3 days with various concentrations of leptofuranins and then the growth was measured by the MTT method.

Fig. 3. Effect of leptofuranin D on the morphology of normal and transformed rat glia cells.



Cells were cultured for 3 days with or without 100 ng/ml of leptofuranin D.

were separately concentrated to dryness to give colorless oils of leptofuranins C (15 mg) and D (24 mg).

The second fraction of the first silica gel column was rechromatographed on a silica gel column (40 ml) with hexane - ethyl acetate (1 : 1). The active eluate was subjected to silica gel TLC with chloroform - methanol (20 : 1) followed by reverse-phase HPLC (YMC-Pack D-ODS-7, Yamamura Chemical Laboratories Co.). Development of the column with 75% methanol gave two active fractions, which were separately evaporated to dryness to yield colorless oils of leptofuranins A (80 mg) and B (56 mg).

Biological Activity

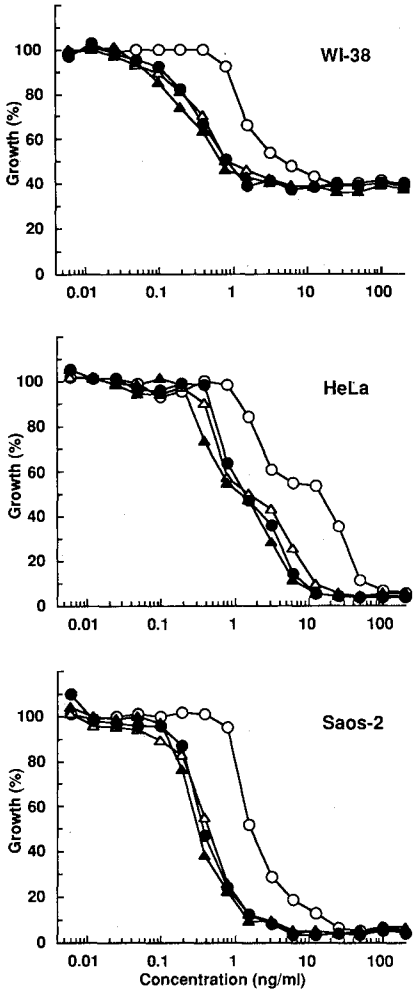
The cytostatic and cytotoxic effects of leptofuranins A to D on normal and transformed cells were examined by using rat glia cells and glia cells transformed with E1A or both E1A and E1B genes (RG-E1A-7 and RG-E1-4 cells). At very low concentrations, the leptofuranins arrested the growth of normal cells and caused cell death against transformed cells as shown in Figs. 2 and 3. Cell death induced by leptofuranins was not inhibited by E1B anti-apoptotic gene products.

The effects of the leptofuranins were further investigated using human cells. As shown in Fig. 4, leptofuranins A to D arrested the growth of WI-38 normal human fibroblasts, and induced cell death against human cervical cancer HeLa and human osteosarcoma Saos-2 cells, in which pRB is inactivated by the human papillomavirus E7 oncoprotein and the loss of RB gene, respectively.

During apoptosis, loss of membrane integrity is typically preceded by chromatin condensation and internucleosomal cleavage of genomic DNA[15]. Significant numbers of HeLa cells treated with 100 ng/ml of leptofuranin D for 3 days contained condensed chromatin and fragmented nuclei as visualized by staining with Hoechst Dye 33258 (Fig. 5). After treatment with 100 ng/ml of leptofuranin D for 3 days, HeLa cells contained a large amount of degraded DNA, which was not observed in WI-38 normal fibroblasts (Fig. 6). These data suggest that cell death induced by leptofuranins

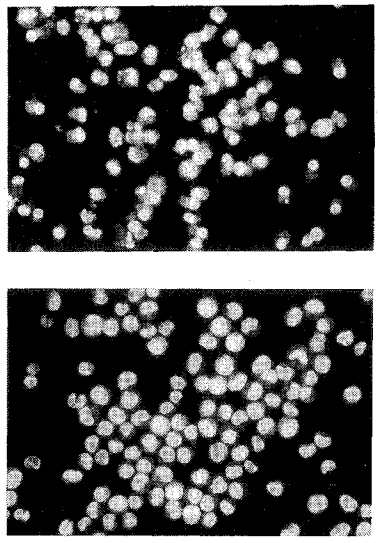
Fig. 4. Effect of leptofuranins A to D on the growth of human cells.

○ Leptofuranin A, △ leptofuranin B, ● leptofuranin C, ▲ leptofuranin D.



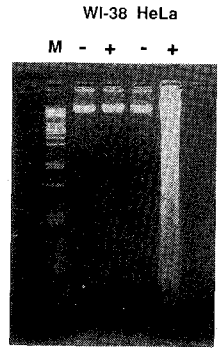
Cells were cultured for 3 days with various concentrations of leptofuranins and then the growth was measured by the MTT method.

Fig. 5. Fluorescence micrographs of HeLa cells stained with Hoechst Dye 33258.



Cells were cultured for 3 days with (top) or without (bottom) 100 ng/ml of leptofuranin D.

Fig. 6. Ethidium bromide-stained DNA extracted from WI-38 or HeLa cells.



M: Marker, −: control, +: leptofuranin D treatment (100 ng/ml, 3 days).

resulted from a programmed cellular response, apoptosis. Further studies on the biological activities of leptofuranins are in progress.

Acknowledgments

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