J. Antibiot. 59(1): 1–5, 2006

ORIGINAL ARTICLE

THE JOURNAL OF ANTIBIOTICS

Thioviridamide, a Novel Apoptosis Inducer in Transformed Cells from *Streptomyces olivoviridis*

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Received: September 28, 2005 / Accepted: December 13, 2005

Abstract In the course of screening for antitumor antibiotics using 3Y1 rat fibroblasts transformed with adenovirus oncogenes, a new active substance designated thioviridamide was isolated from the culture broth of an actinomycete. The producing organism was identified as *Streptomyces olivoviridis* on the basis of its culture characteristics and physiological properties. Thioviridamide showed cytotoxicity selectively against Ad12-3Y1 cells (IC_{50}=3.9 ng/ml) and E1A-3Y1 cells (IC_{50}=32 ng/ml), both of which contain the adenovirus E1A oncogene. Significant numbers of Ad12-3Y1 cells treated with thioviridamide contained condensed chromatin and fragmented nuclei, indicating that thioviridamide induced apoptosis.

Keywords thioviridamide, antitumor antibiotics, apoptosis, *Streptomyces*, adenovirus oncogene

Introduction

The retinoblastoma tumor suppressor protein (pRB) plays an important role in cell-cycle and apoptosis control in mammalian cells, and is inactivated during the development of a wide variety of human cancers [1, 2]. The adenovirus E1A gene product inactivates pRB thereby stimulating host cell DNA synthesis. However, this function of E1A induces the host cells to undergo apoptosis. For this reason, adenoviruses encode a second function in the E1B gene that prolongs host cell viability by inhibiting apoptosis [3, 4]. At low frequency, rodent cells are transformed by E1A alone, and the stable transformants expressing E1A seem to exhibit an attenuated apoptotic response to E1A [3]. It is clear that pRB-inactivated cells require additional genetic alterations that render them resistant to apoptotic stimuli. E1A/E1B-transformed cells and E1A-transformed cells might be appropriate models for pRB-inactivated tumor cells that suppress apoptosis by known and unknown mechanism, respectively.

In the course of screening for antitumor antibiotics using 3Y1 rat fibroblasts transformed with adenovirus oncogenes, an actinomycete identified as *Streptomyces olivoviridis* was found to produce an active substance designated thioviridamide (Fig. 1), which was determined to be a novel cyclic peptide containing five thioamide groups by NMR spectral analysis. Thioviridamide induced apoptotic cell death selectively in 3Y1 rat fibroblasts transformed with adenovirus type 12 or its E1A gene. This paper describes the fermentation, isolation, physico-chemical properties and biological activities of thioviridamide as well as the taxonomy of the producing organism. The structure elucidation of thioviridamide is described in the accompanying paper [5].

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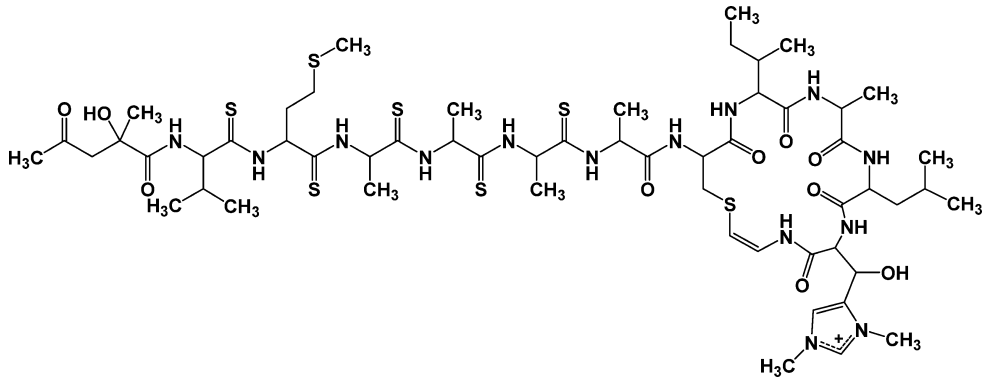

Fig. 1 Structure of thioviridamide.

Materials and Methods

General

UV and IR spectra were measured on Hitachi U-3210 and JASCO FT/IR-470plus spectrometers, respectively. Mass spectra were obtained on a JEOL HX-110 spectrometer in the FAB mode using *m*-nitrobenzyl alcohol as matrix and polyethylene glycol as internal standard. Optical rotations were recorded on a JASCO DIP-1000 spectropolarimeter.

Microorganism

The producing organism designated NA05001 was isolated from a soil sample collected at Ogasawara-mura, Tokyo, Japan. Strain NA05001 was deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, with the name of *Streptomyces olivoviridis* NA05001 under accession No. FERM BP-10289.

Taxonomy

The characterization and identification of the culture were carried out by the method of the International Streptomyces Project (ISP) [6, 7]. For the evaluation of culture characteristics, the strain was incubated at 27°C for 21 days. Cell wall composition was analyzed by the methods of Becker [8].

Cells and Cell Culture

Rat 3Y1 fibroblasts and 3Y1-derived cell lines [9～11] were obtained from the Japanese Cancer Research Resources Bank (JCRB). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum and 0.1% glucose.

MTT Assay

Cells at 50% confluence were plated at one tenth lower cell density and incubated for 3 days with various concentrations of a sample. The growth was measured by formazan formation (detected at 570 nm) 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. IC_{50} values were calculated from the concentration-inhibition response curves. The IC_{50} values were calculated by linear interpolation between the two drug concentrations above and below the 50% inhibition line.

Apoptosis Assay

Cells were treated with 0.4 μg/ml of thioviridamide for 18 hours, and adherent and nonadherent cells were pooled. Chromatin structure was visualized by fluorescence microscopy after staining fixed cells (1×10^6 cells) with Hoechst Dye 33258.

Results and Discussion

Identification of the Producing Organism

The aerial mycelium of culture NA05001 irregularly branched on the long main stem and terminated in straight spore chains, hooked spore chains or rarely in compact loops, forming spore chains with 10～50 spores per chain. The spores were oval in shape (0.4～0.6×1.0～1.4 μm) with a spiny surface. Whole-cell analysis showed that the strain contained L,L-diaminopimelic acid, indicating cell-wall type I. The culture characteristics and physiological properties of NA05001 are summarized in Tables 1 and 2, respectively. On the basis of these characteristics, strain NA05001 seems to belong to the genus *Streptomyces*. Among the species of *Streptomyces* described in Shirling's reports [12～15], these properties resemble those of

Table 1 Culture characteristics of strain NA05001

Agar medium	Aerial mycelium	Substrate mycelium	Soluble pigment
Sucrose - nitrate	Gray-color series	Pale yellow	None
Glucose - asparagine	Gray-color series	Pale yellowish brown	None
Glycerol - asparagine	Gray-color series	Pale yellowish brown	None
Inorganic salts - starch	Gray-color series	Pale yellowish brown	None
Tyrosine	Gray-color series	Dark brownish gray	Pale yellowish brown
Nutrient	No aerial mycelium	Pale yellow	None
Yeast extract - malt extract	Gray-color series	Yellowish brown to pale yellowish brown	Pale yellowish brown
Oatmeal	Gray-color series	Light yellow to pale yellowish brown	None

Table 2 Comparison of strain NA05001 with *Streptomyces olivoviridis*

	NA05001	*Streptomyces olivoviridis*[a]
Spore chain morphology		
Straight	+	+
Hooks	+	+
Loops	+	+
Spore surface		
Spiny	+	+
Color of colony		
Gray	+	+
Reverse color		
Distinctive	+	+
Olive	+	+
pH sensitivity	−	−
Soluble pigment		
Production	+	+
pH sensitivity	−	−
Melanoid pigment	+	+
Hydrolysis of starch	+	+
Nitrate reduction	−	−
Growth at 45°C	−	−
Utilization of carbon source		
L-Arabinose	+	+
D-Xylose	+	+
i-Inositol	+	−
D-Mannit	+	+
L-Rhamnose	+	+
Raffinose	+	−
Sucrose	+	−
D-Fructose	+	+

[a] Properties have been reported [13].

Streptomyces olivoviridis. A comparison of strain NA05001 with *S. olivoviridis* [13] as shown in Table 2 gave a good agreement except for carbon utilization, and therefore, culture NA05001 was identified as a strain of *Streptomyces olivoviridis*.

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 Bennett'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 a production medium consisting of glucose 2.5%, fish meal 1.5%, dry yeast 0.2% and calcium carbonate 0.4% (pH 7.2). The fermentation was carried out on a rotary shaker at 27°C for 4 days.

Isolation

The fermentation broth (2 liters) was centrifuged and the mycelium was extracted with acetone. After evaporation, the aqueous concentrate was extracted with ethyl acetate at pH 5.0. The extract was applied to a silica gel column, which was washed with chloroform - methanol (10 : 1) and eluted with chloroform - methanol (2 : 1). The active eluate was subjected to HPLC (YMC Pack D-ODS-7, 20×250 mm) with 85% methanol containing 5 mM disodium hydrogen citrate. The active fraction was concentrated and then partitioned between ethyl acetate and water. The organic layer was successively washed with water, 0.01 M hydrochloric acid and water. The ethyl acetate solution was dried over anhydrous sodium sulfate and evaporated to dryness to give a chloride salt of thioviridamide (2.7 mg).

Table 3 Physico-chemical properties of thioviridamide chloride

Appearance	Colorless powder
MP	146～150°C
$[\alpha]_D^{23}$	−146° (*c* 0.285, MeOH)
Molecular formula	$C_{56}H_{93}N_{14}O_{10}S_7Cl$
HR-FAB-MS (*m/z*)	
Found	1345.5215 $(M-Cl)^+$
Calcd.	1345.5244
UV λ_{max}^{MeOH} nm (ε)	247 (sh. 29,000), 274 (31,500)
IR ν_{max} (KBr) cm^{-1}	3350, 1710 (sh.), 1670, 1530

Table 4 Antitumor activity of thioviridamide against transformed rat fibroblasts

Cell line	Oncogene	IC_{50} (ng/ml)
3Y1		890
Ad12-3Y1	E1A, E1B	3.9
E1A-3Y1	E1A	32
SR-3Y1	v-*src*	630
SV-3Y1	SV40 large T antigen	460
HR-3Y1	v-H-*ras*	200

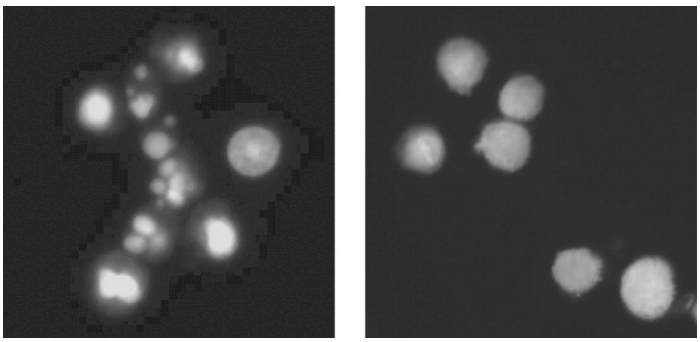

Fig. 2 Fluorescence micrographs of Ad12-3Y1 cells stained with Hoechst Dye 33258. Cells were cultured for 18 hours with (left) or without (right) 0.4 μg/ml of thioviridamide.

Physico-chemical Properties

The physico-chemical properties of thioviridamide chloride are summarized in Table 3. The molecular formula of thioviridamide was established to be $C_{56}H_{93}N_{14}O_{10}S_7^+$ (ion) by high-resolution FAB-MS. The IR spectrum of thioviridamide chloride indicates the presence of ketone (1710 cm^{-1}) and amide (1670 cm^{-1}) groups.

Biological Activity

The antitumor activity of thioviridamide was examined by using 3Y1 rat normal fibroblasts and 3Y1 cells transformed with adenovirus type 12 (Ad12-3Y1), adenovirus E1A gene (E1A-3Y1), SV40 (SV-3Y1), v-*src* (SR-3Y1) or v-H-*ras* (HR-3Y1) [9～11]. Thioviridamide showed selective cytotoxicity against Ad12-3Y1 cells (IC_{50}=3.9 ng/ml) and E1A-3Y1 cells (IC_{50}=32 ng/ml), both of which contain the adenovirus E1A oncogene (Table 4). At higher concentrations, thioviridamide inhibited the growth of the other cell-lines including normal fibroblasts without distinct cell death. The cytotoxicity of thioviridamide seems to not be affected by a typical growth stimulator such as Ras or Src. During apoptosis, loss of membrane integrity is typically preceded by chromatin condensation and nuclear fragmentation [16]. Significant numbers of Ad12-3Y1 cells treated with 0.4 μg/ml of thioviridamide for 18 hours contained condensed chromatin and fragmented nuclei as visualized by staining with Hoechst Dye 33258 (Fig. 2), suggesting that cell death induced by thioviridamide resulted from a programmed cellular response, apoptosis. Since E1B-containing Ad12-3Y1 cells were highly sensitive to thioviridamide, the E1B anti-apoptotic gene appears to not prevent cell death induced by thioviridamide. Interestingly, thioviridamide did not induce apoptosis in SV-3Y1 cells, although SV40 large T antigen can inactivate pRB in a manner like E1A. It has been reported that E1A can induce apoptosis in pRB-deficient cells [17]. Thioviridamide might stimulate the apoptotic signal from E1A independent of pRB. These results suggested that thioviridamide is a possible candidate for an anticancer agent against tumor cells expressing an E1A-like oncogene. Further studies on the biological activities of thioviridamide are in progress.

Acknowledgements This work was supported in part by a Grant-in-Aid for Scientific Research, The Ministry of Education, Science, Sports and Culture, Japan.

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