A New Differentiation Inducer of Friend Leukemia Cells, Trichostatic Acid

Hajimu MORIOKA, Masaru ISHIHARA, Misako TAKEZAWA,
Kazuo HIRAYAMA, Eiichiro SUZUKI, Yasuo KOMODA*
and Hiroshiro SHIBAI

*Central Research Laboratories, Ajinomoto Co., Ltd.,
1-1 Suzuki-cho, Kawasaki-ku, Kawasaki 210, Japan*

**Research Institute for Medical and Dental Engineering,
Tokyo Medical and Dental University,
2-3-10 Surugadai, Kanda, Chiyoda-ku,
Tokyo 110, Japan*

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FL-657B, which induced differentiation of Friend leukemia cells, was isolated from the culture fluid of *Streptomyces sioyaensis* and identified with trichostatic acid, a hydrolysis product of trichostatin A and C. FL-657B induced hemoglobin biosynthesis of both dimethyl sulfoxide-sensitive and -resistant Friend leukemia cells. FL-657B caused approximately 90% of the cells to be benzidine positive and reduced the growth to approximately 30~70% of the control at 2.42 $\mu\text{g/ml}$. Hemoglobin newly biosynthesized by the induction of FL-657B showed a UV absorption pattern similar to that from the normal mouse.

In searching for new antitumor antibiotics, we have developed an assay system for tumor differentiation employing Friend leukemia cells, and have applied it to a wide variety of culture fluids of streptomyces. As a result, we found that the initiation of hemoglobin biosynthesis was induced by FL-657, which was produced by *Streptomyces sioyaensis* and consisted of more than two active compounds.

The present paper reports the isolation of one component FL-657B, and its identification with trichostatic acid.

MATERIALS AND METHODS

Cell lines and culture conditions. Friend leukemia cells, F5-5 and C9-6, were kindly provided by Ikawa, who established them from the DDD mouse.¹⁾ F5-5 differentiated to synthesize hemoglobin with the stimulation of DMSO, but C9-6 did not. The Friend leukemia cell, C1745A, originated from DBA/2 mouse²⁾ and was a gift from Onodera. The culture media were Ham's F-12 supplemented with 10% fetal calf serum for F5-5 and C9-6, and Dulbecco's MEM supplemented with 10% fetal calf

serum for C1745A.

The cells were cultured at 37°C for 6 days at initial cell densities of 2×10^4 cells/ml for F5-5 and C9-6, and at 1×10^5 cell/ml for C1745A. The culture volume was 0.2 ml, to which 0.02 ml of microbial culture fluids or their diluted solution were added. The Cell cultures were maintained in a humidified incubator in an atmosphere of 95% air and 5% CO₂.

Cell density was measured by the trypan blue dye exclusion method and counted on a Burkert-Turk counter.

Differentiation of Friend leukemia cells was assayed by the benzidine staining method.³⁾ On the 6th day, 0.2 ml of the cell suspension was mixed with 0.02 ml of freshly prepared benzidine solution (a 10 to 1 mixture of 2% 3,3'-dimethoxy-benzidine in 0.5M acetic acid and 30% hydrogen peroxide). The cells that were stained blue, indicating the presence of hemoglobin, were scored in a hemacytometer as the percentage of the total cell number.

Colony formation in the soft agar medium. In order to examine the cell's plating efficiency, a colony forming assay was carried out. The F5-5 cells were suspended in Ham's F-12 medium supplemented with 10% fetal calf serum to give a cell density of 2×10^3 cells/ml. The cell suspension (4 ml) was mixed with 16 ml of an agar medium consisting of 0.35 ml of 7.5% NaHCO₃, 11.25 ml of Ham's F-12 medium concentrated by twice, 2.5 ml of fetal calf serum, 3.75 ml of 3% agar (Difco) and 7.15 ml of water.

This cell suspension (0.5 ml) in the agar medium was overlaid on 0.5 ml of the agar medium in a 24 well dish. On the solidified agar medium, 1 ml of a liquid medium containing 0.2 ml of the samples being tested was added. The soft agar culture was carried out at 37°C for 10–14 days, and the colonies formed were counted.

Production of FL-657B. The producer of FL-657 was isolated from soils and identified with *Streptomyces sioyaensis*. The culture medium comprised of 2.0% soluble starch, 1.0% glucose, 0.2% yeast extract, 0.7% bacto-soytone, 0.5% bacto-peptone, 0.1% KH_2PO_4 and 0.1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The organism was cultured in 100 ml medium in a 500 ml shaking flask at 27°C for 3 days. A 300 liter fermentation was carried out employing 20 liters of the culture broth as an inoculum at 27°C for 3 days. The agitation speed was 310 rpm and aeration rate 1/2 vvm.

The culture broth was separated into mycelium and culture supernatant by centrifugation.

Differentiation inducing activities of the mycelium and culture supernatant were then measured. The mycelium was separated from 10 ml of the culture broth and extracted with 10 ml of chloroform–methanol (1:1). The extracts were evaporated and again dissolved in 1 ml of methanol. Serial 1:2 dilutions of the mycelium extracts and supernatants were made in a 96-well plate. Friend leukemia cell, F5-5 was cultured with a serially diluted test sample at a concentration of 10% for 6 days. The titer of the sample expressed as units/ml of the extract or supernatant was defined as the reciprocal of the maximum dilution necessary to induce benzidine positive cells by at least 1%.

Analysis. The UV spectrum was taken with a Hitachi 100-60 spectrophotometer, IR spectrum with a DIGILAB FTS-15E spectrophotometer, field desorption (FD) mass spectrum with a JEOL JMS-DX-300 and ^1H -NMR and ^{13}C -NMR spectra with a JEOL JMN-GX-400.

Materials. Mammalian cell culture media and fetal calf serum were obtained from Flow Lab., other chemicals being obtained from commercial sources.

RESULTS

Production and isolation of FL-657B

The microorganism producing FL-657B was identified with *Streptomyces sioyaensis*. A large scale fermentative production of FL-657B was carried out using a 400 liters jar fermentor. The differentiation inducing activity was observed in both the mycelium and culture supernatant of *Streptomyces sioyaensis*. The activity present in the mycelium and culture supernatant reached maxima after 3 and 2 day's cultivation, respectively.

The culture supernatant (260 liters) after 3 days cultivation was applied to an Amberlite XAD-7 resin column, eluted with methanol and concentrated *in vacuo*.

The mycelium (13 kg on a wet basis) separated from the same culture broth was extracted with chloroform–methanol (1:1). The active extract was concentrated to a syrup (0.5 liters).

The active fractions obtained from the mycelium and culture supernatant were combined and extracted twice with ethyl acetate. The ethyl acetate layer was evaporated *in vacuo*, and the residue was dissolved in methanol and applied to an LH-20 column followed by elution with methanol.

The active fractions were dissolved in acetonitrile–10 mM acetic acid (35:65, pH 4.0) and aliquots were applied to preparative high-performance liquid chromatography (HPLC) using a reverse-phase column (μ Bondapack C18, Waters), and eluted with the same solvent.

The active eluate was further purified by a second preparative HPLC using a solvent system of acetonitrile–10 mM acetic acid (32.5:67.5, pH 4.0). The combined active eluate was extracted twice with ethyl acetate. The ethyl acetate layer was washed three times with water, dried over anhydrous sodium sulfate and evaporated to dryness to give 12 mg of the active substance, FL-657B.

Identification of FL-657B

FL-657B was found to have the molecular formula, $\text{C}_{17}\text{H}_{21}\text{NO}_3$, by high resolution mass spectrometry and elemental analysis. The UV spectrum in methanol showed its absorption maxima at 250 and 340 nm. A carbonyl group (1662 cm^{-1}), benzene ring (1597 cm^{-1}) and carboxylic acid group (1566 cm^{-1}) were detected in the IR spectrum of FL-657B with KBr. The ^1H - and ^{13}C -NMR spectral data are shown in Tables I and II, respectively.

These spectral data and the results obtained by spin decoupling and nuclear Overhauser effect experiments in H-NMR revealed that FL-657B has the structure as shown in Fig. 1.

TABLE I. ^1H -NMR SPECTRAL DATA OF FL-657B

The ^1H -NMR spectrum of FL-657B was taken in CDCl_3 . Chemical shifts in ppm are downfield from internal TMS. s, singlet; d, doublet; dq, double quartets; J , coupling constants in Hz.

Proton	Assignment
2'-CH ₃	1.32 (3H, d, $J=6.8$)
4'-CH ₃	1.93 (3H, d, $J=1.2$)
4-N(CH ₃) ₂	3.06 (6H, s)
2'-H	4.40 (1H, dq, $J=6.8, 9.5$)
6'-H	5.83 (1H, d, $J=15.4$)
3'-H	6.09 (1H, dq, $J=1.2, 9.5$)
3- and 5-H	6.65 (2H, d, $J=9.0$)
5'-H	7.38 (1H, d, $J=15.1$)
2 and 6-H	7.85 (2H, d, $J=9.3$)

TABLE II. ^{13}C -NMR SPECTRAL DATA OF FL-657B

The ^{13}C -NMR spectrum of FL-657B was taken in CDCl_3 . Chemical shifts in ppm are downfield from internal TMS. s, singlet; d, doublet; q, quartet.

Carbon	Chemical shift (ppm)
4'-CH ₃	12.5 (q)
2'-CH ₃	17.7 (q)
N-(CH ₃) ₂	40.0 (q) $\times 2$
2'	40.9 (d)
3, 5	110.8 (d) $\times 2$
6'	115.6 (d)
1	123.9 (s)
2, 6	130.7 (d) $\times 2$
4'	132.6 (s)
3'	143.0 (d)
5'	151.4 (d)
4	153.6 (s)
7'	171.3 (s)
1'	198.3 (s)

FIG. 1. Chemical Structure of FL-657B (Trichostatic Acid).

This compound has already been reported by Tsuji *et al.*^{4,5)} as trichostatic acid, a hydrolysis product of trichostatin A and C, which were isolated as antifungal antibiotics from *Streptomyces hygroscopicus* Y-50.

FL-657B was identified as trichostatic acid by direct comparison with an authentic sample.

Differentiation inducing activity of FL-657B

FL-657B showed differentiation inducing activity to F5-5 cells in a concentration range from 0.3 to 5 $\mu\text{g}/\text{ml}$ as shown in Fig. 2. Its activity was extremely potent and as much as 90% of the cells were induced to biosynthesize hemoglobin at 3 $\mu\text{g}/\text{ml}$. It was also cytotoxic to the F5-5 cells, showing an ED value of approximately 4 $\mu\text{g}/\text{ml}$.

Figure 2 also shows FL-657B's strong inhibition of the colony formation of Friend leukemia cells in the soft agar medium at such a concentration that did not cause the growth inhibition in the liquid medium or the induction of hemoglobin biosynthesis. The result indicated that the loss of the plating efficiency followed the induction of hemoglobin biosynthesis.

The newly biosynthesized hemoglobin in the F5-5 cells had an absorption at 415, 540 and 577 nm as in the case of mouse hemoglobin,⁶⁾ suggesting that FL-657B has an activity to introduce the Friend leukemia cell, F5-5, into the normal erythroid path-way.

Mode of action of FL-657B on differentiation

The effect of FL-657B on the differentiation of the F5-5 cells was compared with that of DMSO (Table III). When the F5-5 cells were incubated in the presence of FL-657B for 8 hr, they could initiate the biosynthesis of hemoglobin, and approximately 10% of the cells were hemoglobin-positive when successively cultured for 48 hr in the absence of FL-657B. However, at least 24 hr were required for DMSO to induce 14.4% of hemoglobin-positive cells.

FL-657B was active as an inducer not only to F5-5 but also to C9-6 and C1745A (Table IV). As much as 95.8% of the C9-6 cells were induced to biosynthesize hemoglobin by FL-657B at a concentration of 2.42 $\mu\text{g}/\text{ml}$, while only 5.8% of the C9-6 cells were induced by DMSO at a concentration of 20 mg/ml.

FIG. 2. Effect of FL-657B Dose on Growth in the Liquid and Soft Agar Media, and the Differentiation of Friend Leukemia Cell, F5-5.

The growth of the cells in the liquid and soft agar media containing FL-657B was compared with that in control cultures. —○—, relative survival cell number in the liquid medium; —□—, relative colony number in the soft agar medium; —●—, benzidine positive cells.

TABLE III. EFFECT OF THE TIME OF EXPOSURE TO INDUCERS ON GROWTH AND DIFFERENTIATION

The F5-5 cells were cultured in 25 ml of medium in the presence of FL-657B (1.8 µg/ml) or DMSO (20 mg/ml). At the time indicated, the cultures (5 ml) were centrifuged and washed twice with saline phosphate buffer, and resuspended in 5 ml of fresh medium without the inducers. At a sampling time counted from the initiation of the cultures, 0.2 ml of the cell suspension was taken out and the cell growth and differentiation were scored.

Compound	Contact period (hr)	Cell density ($\times 10^4$ cells/ml)				Benzidine positive cells (%)			
		Sampling time (hr)				Sampling time (hr)			
		24	48	72	144	24	48	72	144
FL-657B	0	14.2	50.0	69.3	58.7	0	0	0	0
	8	6.7	32.9	54.7	53.8	0	9.8	7.3	4.0
	24	7.1	12.2	34.4	62.2		50.0	56.1	16.0
	30		9.1	14.4	28.2		61.8	74.5	20.7
	48		6.2	11.8	4.4		28.6	68.9	25.8
	72			10.4	0.7			64.2	60.9
	144				3.8				43.8
DMSO	0	11.8	52.0	66.7	54.7	0	0	0	0
	8	10.8	39.8	70.9	60.0	0	1.8	1.8	1.4
	24	11.3	20.0	62.4	54.7		14.4	13.0	4.7
	30		16.7	62.9	54.9		19.7	15.4	11.5
	48		22.2	48.4	60.7		14.2	47.4	35.1
	72			44.0	80.4			40.0	48.8
	144				36.0				62.2

TABLE IV. DIFFERENTIATION INDUCING ACTIVITY OF FL-657B AGAINST THREE DIFFERENT FRIEND LEUKEMIA CELLS

The differentiated cells were counted at 6 days.

Cell line	Compound conc. ($\mu\text{g/ml}$)	Cell density ($\times 10^4$ cells/ml)	Benzidine positive cells (%)
F5-5	FL-657B	0	0
		0.61	9.6
		1.21	37.3
		2.42	87.5
		4.84	84.4
		9.69	ND
	DMSO	5.0×10^3	3.8
		10.0×10^3	24.6
		20.0×10^3	77.6
C9-6	FL-657B	0	0
		0.61	6.7
		1.21	46.1
		2.42	95.8
		4.84	76.7
		9.69	ND
	DMSO	5.0×10^3	0
		10.0×10^3	0
		20.0×10^3	5.8
C1745A	FL-657B	0	0.5
		0.61	0.5
		1.21	13.5
		2.42	18.9
		4.84	16.4
		9.69	ND
	DMSO	5.0×10^3	2.6
		10.0×10^3	20.5
		20.0×10^3	50.6

ND, not determined.

However, FL-657B was weak as an inducer to the C1745A cells, bringing about only 18.9% of the hemoglobin-positive cells at the most, compared with 50.6% for DMSO.

DISCUSSION

FL-657B, which was isolated from the culture fluid of *Streptomyces sioyaensis* by employing the Friend leukemia cell differentiation assay system, was identified with trichostatic acid. Trichostatic acid is a hydrolysis product of trichostatin A and C as reported by Tsuji *et al.*^{4,5)} Trichostatic acid may be an intermediate in the biosynthesis of trichostatin A and C. Tsuji *et al.* found trichostatin A and C in the culture broth of *Streptomyces hy-*

groscopicus, and observed their antifungal activity against *Aspergillus niger*; however, the biological activity of trichostatic acid was not reported.

This report is the first to describe the isolation of trichostatic acid from a microbial culture, as well as its differentiation inducing activity against Friend leukemia cells.

Although the mechanism of action of FL-657B is not yet understood, the activity of FL-657B seems to be potent and specific compared with that of DMSO from the following results: (1) The incubation time required with FL-657B for the induction was short compared with that of DMSO. (2) The concentrations of the drugs were $2 \mu\text{g/ml}$ for FL-657B and 20 mg/ml for DMSO. (3) FL-657B was also

cytotoxic to the Friend leukemia cells but DMSO was not. (4) FL-657B could induce differentiation of the DMSO-insensitive C9-6 cells. FL-657B may have different primary sites of action from DMSO.

According to Marks *et al.*,^{7~12)} planer-polar compounds like hexamethylene bisacetamide have a strong differentiation inducing activity due to their interaction with the plasma membrane and with DNA. The polymethylene chain joining the junctional groups is 5 to 6 carbon atoms in length to achieve maximum activity. FL-657B also possesses a 5-carbon chain with two double (*trans*) bonds between the functional carboxylic acid and *p*-dimethylaminobenzoyl groups. This indicates that FL-657B has a similar mechanism of action to hexamethylene bisacetamide. However, in spite of the structural similarity, FL-657B may be a much more potent inducer than hexamethylene bisacetamide. The drug concentration required for differentiation was 5~20 μ M for FL-657B, compared with 1~5 mM for hexamethylene bisacetamide, according to the report.¹⁰⁾ This suggests that FL-657B is much more likely to interact with active centers concerning the induction of differentiation of Friend leukemia cells. The active centers may be genes essential in multi-step transcriptions for differentiation.^{11,13)} Elucidation of the mechanism of action of FL-657B in Friend leukemia cells should shed light on the differentiation process.

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