A NEW ANTITUMOR SUBSTANCE, BE-13793C, PRODUCED BY A STREPTOMYCETE

TAXONOMY, FERMENTATION, ISOLATION, STRUCTURE DETERMINATION AND BIOLOGICAL ACTIVITY

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(Received for publication February 28, 1991)

A new antitumor substance, BE-13793C, which has topoisomerase inhibitory activity was isolated from the culture broth of a strain of actinomycetes. The producing strain, BA13793, isolated from a soil sample collected in Seto, Aichi Prefecture, Japan, had a resemblance to *Streptoverticillium mobaraense*. The active principle was extracted from the mycelium of strain BA13793 with methanol and purified by Sephadex LH-20 column chromatography. BE-13793C showed strong inhibitory activity against topoisomerases I and II and inhibited the growth of doxorubicin-resistant or vincristine-resistant P388 murine leukemia cell lines, as well as their parent P388 cell line.

In the course of our screening program for new antitumor substances, strain BA13793 was found to produce an active component designated BE-13793C which inhibited topoisomerases I and II. BE-13793C inhibited the growth of doxorubicin-resistant (P388/ADR) or vincristine-resistant (P388/VCR) P388 murine leukemia cell lines. This paper describes the taxonomy of the producing strain and the fermentation, isolation, structure determination and biological properties of BE-13793C. The structure of BE-13793C is shown in Fig. 1.

Taxonomy of the Producing Organism

The producing organism, strain BA13793, was isolated from a soil sample collected in Seto, Aichi Prefecture, Japan. Characterization of the strain followed the method adopted by the International Streptomyces Project (ISP)[1] and several other tests were also used. Morphological observation of the culture showed that both substrate and aerial mycelia were well-branched and non-fragmented. The aerial mycelium exhibited a typical whorl formation. The spore chains had five to ten spores, ranging in size from 0.5×1 to $1.5\,\mu m$, and the spore surface was smooth. The color of the aerial mycelium was grayish white to light gray after sporulation. The reverse side of the growth was pale yellowish brown. Melanin and other soluble pigments were not produced (Table 1). The physiological properties

Fig. 1. The structure of BE-13793C.

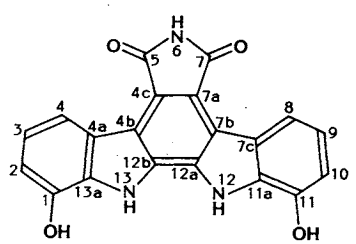


Table 1. Cultural characteristics of strain BA13793.

Agar medium	Growth	Aerial mycelium	Reverse	Soluble pigment
Yeast extract-malt extract agar (ISP-2)	Good	Thin Grayish white Cottony	Pale yellowish brown	None
Oatmeal agar (ISP-3)	Good	Abundant Light gray Cottony	Pale orange	None
Inorganic salts-starch agar (ISP-4)	Good	Abundant Light gray Cottony	Pale yellowish brown	None
Glycerol-asparagine agar (ISP-5)	Good	Abundant Grayish white Powdery	Yellowish orange	None
Peptone-yeast extract-iron agar (ISP-6)	Good	Abundant Grayish white Powdery	Pale yellowish brown	None
Tyrosine agar (ISP-7)	Good	Abundant Grayish white Powdery	Pale yellowish brown	None
Nutrient agar	Good	Abundant Light gray Powdery	Pale yellowish brown	None
Sucrose-nitrate agar	Poor	Thin Colorless	Colorless	None
Glucose-asparagine agar	Poor	Thin Colorless	Yellowish orange	None

Table 2. Physiological properties of strain BA13793.

		Carbon utilization of	
Melanin formation	Negative	D-Glucose	+
Hydrolysis of starch	Positive	D-Xylose	−
Coagulation of milk	Negative	L-Arabinose	−
Peptonization of milk	Negative	L-Rhamnose	±
Liquefaction of gelatin	Negative	D-Fructose	+
NaCl tolerance	4%	D-Galactose	+
Temperature range for growth	12~40°C	Raffinose	+
Optimum temperature	32°C	D-Mannitol	−
		Inositol	+
		Salicin	−
		Sucrose	±

+: Utilized.

and the utilization of carbon sources of the strain are shown in Table 2. The whole cell hydrolysate of the strain contained L,L-diaminopimelic acid and glycine.

Based on its characterization, strain BA13793 is considered to belong to the genus *Streptoverticillium*. According to the descriptions of the species by BALDACCI and LOCCI[2], and SHIRLING and GOTTLIEB[3], it closely resembles *Streptoverticillium mobaraense*. The characteristics of strain BA13793 were compared with those of the standared strain of *S. mobaraense*. As a result, good agreement was obtained, except for NaCl tolerance and utilization of raffinose (Table 3). Therefore, strain BA13793 was identified as a strain of *S. mobaraense*. It has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under accession No. FERM P-10489.

Table 3. Comparison of taxonomic characteristics of strain BA13793 with *Streptoverticillium mobaraense*.

	BA13793	*S. mobaraense* (JCM 4168)		BA13793	*S. mobaraense* (JCM 4168)
Spore surface	Smooth	Smooth	Hydrolysis of starch	Positive	Positive
Aerial mass color	Grayish white to light gray	White to light gray	Liquefaction of gelatin	Negative	Negative
Color of reverse	Pale yellowish brown	Pale yellowish brown	Carbon source utilization:		
Soluble pigment	None	None	D-Glucose	+	+
Melanin formation (ISP-7)	Negative	Negative	L-Rhamnose	±	±
Growth at 45°C	No growth	No growth	Raffinose	+	−
NaCl tolerance	No growth on 7% NaCl	Good growth on 7% NaCl	D-Mannitol	−	−
			Inositol	+	+
			Sucrose	±	±

JCM: Japan Collection of Microorganisms, RIKEN (The Institute of Physical and Chemical Research).
+: Utilized.

Fermentation

Four 500-ml conical flasks each containing 100 ml of a culture medium (pH 6.7) comprising glucose 0.1%, dextrin 2.0%, corn gluten meal 1.0%, fish meal 0.5%, yeast extract 0.1%, sodium chloride 0.1%, magnesium sulfate 0.05%, calcium chloride 0.05%, ferrous sulfate 0.0002%, cupric chloride 0.00004%, manganese chloride 0.00004%, cobalt chloride 0.00004%, zinc sulfate 0.00008%, sodium borate 0.00008%, ammonium molybdate 0.00024% and 3-(*N*-morphorino)propanesulfonic acid 0.5% were inoculated with *Streptoverticillium* sp. BA13793 strain grown on agar slant medium. These flasks were then incubated on a rotary shaker (180 rpm) at 28°C for 72 hours.

One ml aliquot of each culture was inoculated into 50 conical flasks of 500 ml capacity, each containing 100 ml of the above mentioned medium and incubated on a rotary shaker (180 rpm) at 28°C for 120 hours.

Isolation

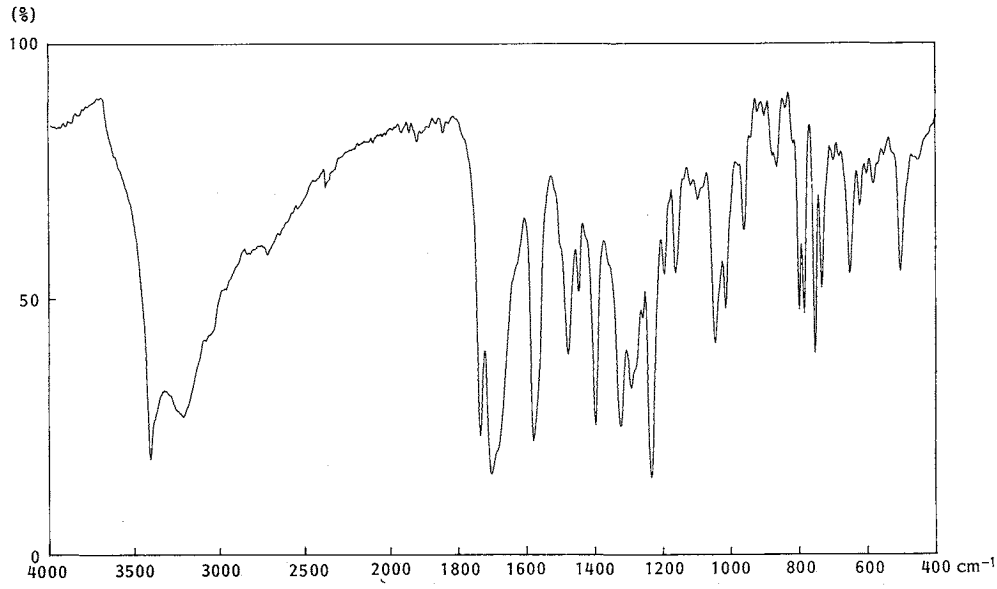
The resulting culture broth (about 5 liters) was filtered and the mycelium obtained was washed with 500 ml of deionized water. Then, 2.5 liters of methanol was added to the mycelium and the mixture was stirred at room temperature for 1 hour. After filtering, a methanol extract was obtained. The extraction with methanol was repeated. The methanol extract (about 5 liters) was combined and concentrated to about 800 ml. The concentrate thus obtained was extracted with 3 liters of ethyl acetate and the ethyl acetate extract was concentrated to dryness. The residue obtained was washed with 500 ml of chloroform. Thus, 720 mg of a crude product containing BE-13793C was obtained. This crude product was dissolved in 2 liters of methanol and concentrated. The orange precipitate thus formed was filtered to give 546 mg of a product containing BE-13793C. The product was dissolved in a solvent mixture (methanol-tetrahydrofuran, 1:1) and subjected to column chromatography with the use of Sephadex LH-20 (1.5 × 120 cm, Pharmacia) and developed with methanol-tetrahydrofuran (1:1). The BE-13793C fraction thus obtained was concentrated to give 99 mg of BE-13793C in the form of a yellowish crystalline substance.

Physico-chemical Properties

BE-13793C was obtained as a yellowish orange crystalline powder. BE-13793C was hardly soluble in water, moderately soluble in methanol and soluble in tetrahydrofuran or dimethyl sulfoxide (DMSO).

BE-13793C possessed the following physico-chemical constants: MP; showing no obvious mp up to

Fig. 2. The IR spectrum of BE-13793C in KBr.



295°C; Rf 0.45 (chloroform-methanol (5:1), Kieselgel 60, Merck); UV λ_{max}^{MeOH} nm (ε) 245 (63,500), 298 (38,100), 307 (37,400), 325 (sh, 21,900), 430 (5,600); HRFAB-MS *m/z* Found: 357.0717 (M, $C_{20}H_{11}N_3O_4$, Calcd: 357.0750). The IR spectrum is shown in Fig. 2. ^{1}H NMR (300 MHz, DMSO-d_6) δ 6.98 (2H, br d, J=7.6 Hz), 7.13 (2H, t, J=7.6 Hz), 8.43 (2H, br d, J=7.6 Hz), 10.20 (2H, br s), 10.88 (1H, br s), 11.59 (2H, br s). ^{13}C NMR (75 MHz, DMSO-d_6) δ 110.9 (d), 115.2 (d), 115.5 (s), 119.7 (s), 120.6 (s), 123.0 (s), 128.7 (s), 130.0 (s), 143.3 (s), 171.2 (s).

Structure Determination

In ^{1}H NMR spectrum, the integral of the signal at 10.88 ppm was half of other signals. The number of carbon atoms observed in ^{13}C NMR was half of that observed in HRFAB-MS. These results indicated that BE-13793C had a symmetrical structure. Acetylation of BE-13793C gave a diacetyl derivative whose ^{1}H NMR spectrum showed a proton signal equivalent to six protons at 2.52 ppm and the signal at 10.20 ppm observed in the ^{1}H NMR spectrum of BE-13793C disappeared. The UV spectrum of the diacetyl derivative of BE-13793C, UV λ_{max}^{MeOH} nm 236, 255 (sh), 283, 311, 395 (sh), was quite similar to that of rebeccamycin[4]. Therefore the existence of the indolopyrrolo carbazole skeleton was suggested. The comparison of the ^{1}H and ^{13}C NMR with those of rebeccamycin and the long range selective proton decoupling (LSPD) experiments of BE-13793C supported the structure of BE-13793C as 12,13-dihydro-1,11-dihydroxy-5*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-5,7(6*H*)-dione as shown in Fig. 1. In the LSPD experiments, 2-H (6.98 ppm) was coupled to C-4 (115.2 ppm) and C-13a (130.0 ppm), 3-H (7.13 ppm) was coupled to C-1 (143.3 ppm) and C-4a (123.0 ppm), 4-H (8.43 ppm) was coupled to C-2 (110.9 ppm), C-4b (115.5 ppm) and C-13a (130.0 ppm), 13-H (11.59 ppm) was coupled to C-12b (128.7 ppm), C-13a (130.0 ppm), C-4a (123.0 ppm) and C-4b (115.5 ppm), 6-H (10.88 ppm) was coupled to C-5 (171.2 ppm) and C-4c (119.7 ppm). Thus obtained assignments of signals in ^{13}C and ^{1}H NMR spectra are shown in Table 4.

Biological Activity

Topoisomerase II activity was measured by the DNA relaxation assay, based essentially on the method

Table 4. ^{13}C and ^{1}H NMR chemical shift data for BE-13793C.

Position	Shift[a]		Position	Shift[a]	
	^{13}C	^{1}H		^{13}C	^{1}H
1	143.3	10.20 (OH)	7c	123.0	—
2	110.9	6.98	8	115.2	8.43
3	120.6	7.13	9	120.6	7.13
4	115.2	8.43	10	110.9	6.98
4a	123.0	—	11	143.3	10.20 (OH)
4b	115.5	—	11a	130.0	—
4c	119.7	—	12	—	11.59 (NH)
5	171.2	—	12a	128.7	—
6	—	10.88 (NH)	12b	128.7	—
7	171.2	—	13	—	11.59 (NH)
7a	119.7	—	13a	130.0	—
7b	115.5	—			

[a] In ppm from TMS in DMSO-d_6.

of MILLER *et al.*[5] using pBR322 plasmid DNA as substrate. Briefly, 20 μl of the reaction mixture consisting of 50 mM Tris-HCl (pH 7.9), 120 mM KCl, 20 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM EDTA, 30 μg/ml bovine serum albumin, 1 mM ATP, 20 μg/ml pBR322 plasmid DNA and 1.5 unit topoisomerase II prepared from L1210 leukemia cells with or without a test sample were incubated at 30°C for 15 minutes. The reaction was stopped by addition of 10 μl of stop solution consisting of 0.1% SDS, 10 mM EDTA and 4% sucrose and the reaction mixture was applied to 1% agarose gel electrophoresis at 8.3 V/cm for 1 hour. The position of DNA in the agarose gel was visualized by staining with ethidium bromide and a picture was taken with a camera. One unit was defined as the minimal amount of the enzyme required to produce full relaxation of supercoiled pBR322 DNA in this assay. Topoisomerase I activity was measured by the same procedure described above, except for omitting ATP from the reaction mixture and using topoisomerase I prepared from L1210 cells as the enzyme.

Table 5. Effect of BE-13793C on Ehrlich ascites tumor.

Substance	Dosage, ip (mg/kg)	MST (day)	T/C (%)
BE-13793C	50	25.8*	197
	20	19.8*	151
	8	16.0	122
Control	—	13.1	100

Inoculum: 10^6 Ehrlich ascites tumor cells were injected intraperitoneally. Host: Female ICR mice. Treatment schedule: BE-13793C was administered once a day from the 1st to the 10th day. * $P<0.05$ by Student's t-test.

BE-13793C inhibited topoisomerases I and II with an IC_{100} value of 2 μM for both enzymes. IC_{100} expresses apparent complete inhibition of DNA-relaxation in the assay described above.

In vitro activity tests were performed for evaluating the inhibitory activities of the antitumor substance BE-13793C on mouse tumor cells. In the *in vitro* antitumor assay using P388 tumor cells, the test substance was first dissolved in DMSO and the solution was serially diluted with a cell culture medium containing 20% of DMSO (20% DMSO-RPMI-1640 medium). Then, 2 μl of the dilution was added to 200 μl of a culture medium (10% fetal calf serum-RPMI-1640 medium) containing 2×10^4 or 3×10^4 tumor cells. The mixture was incubated at 37°C under 5% CO_2 for 72 hours. The viable cells were then counted with a Coulter counter. The results were compared with the control. The antitumor substance BE-13793C showed an intense inhibitory effect on the growth of the P388 tumor cells. The concentration of BE-13793C causing 50% inhibition on P388/S tumor cell growth (IC_{50}) was 0.7 μM, while that on P388/VCR cell growth was also 0.7 μM. The P388/S cells are commonly employed mouse leukemia cells, while the P388/VCR cells are

a subline of P388 cells which have acquired resistance to the antitumor agent vincristine[6]. Furthermore, BE-13793C inhibited the growth of P388/ADR cells, which had acquired resistance to doxorubicin[6], and the IC_{50} thereof was 1.0 μM.

BE-13793C showed an antitumor effect on transplanted mouse Ehrlich tumor cells (ascites type). In this assay, 10^6 tumor cells per mouse were injected intraperitoneally. Then the test substance was serially diluted and administered intraperitoneally. Table 5 summarizes the results. With regard to the acute toxicity of BE-13793C on female ICR mice, no death was found on the 5th day when 100 mg/kg was intraperitoneally administered.

Discussion

An antitumor agent having a new structure is always desired as a new lead for chemotherapeutic agents. Topoisomerases I and II are major targets of currently used clinical antitumor drugs. These drugs, such as amsacrine, doxorubicin, epipodophyllotoxins and camptothecin are thought to stabilize topoisomerase-DNA covalent complexes and thus interfere with DNA replication[7~11]. Because of the efficacy of these drugs and the resistance of tumor cells to them, we have continued screening studies of topoisomerases. BE-13793C described in this report may be a new lead to an antitumor agent.

Acknowledgments

The authors thank Prof. TAKASHI TSURUO of the Tokyo University for generously providing us with the P388/S, P388/VCR and P388/ADR cell lines.

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