ANTITUMOR ACTIVITY OF ECHINOSPORIN

MAKOTO MORIMOTO and RYOJI IMAI

Pharmaceutical Research Laboratory, Kyowa Hakko Kogyo Co., Ltd.,
1188 Shimotogari, Nagaizumi-cho, Sunto-gun, Shizuoka-ken, Japan

(Received for publication September 12, 1984)

Echinosporin isolated from a *Streptomyces* culture showed antitumor activity against rodent tumor models such as leukemia P388, P388/VCR, and fibrosarcoma Meth 1. It was marginally active against melanoma B16 and sarcoma 180. It was not active against Lewis lung carcinoma and xenograft MX-1. It inhibited the colony formation of HeLa S_3 cells with a wide shoulder at low dose ranges. DNA, RNA, and protein synthesis were inhibited by echinosporin. It depressed WBC with nadir on day 3, but the recovery to the normal level after echinosporin injection was more rapid than that after mitomycin C.

Echinosporin with a novel carbon skelton was isolated from the culture broth of *Streptomyces echinosporus* MK-213.[1,2] This antibiotic exhibited weak antibacterial activities against Gram-positive and Gram-negative bacteria. The MICs were 100 μg/ml against *Proteus vulgaris, Salmonella typhosa, Shigella sonnei* and higher than 200 μg/ml against *Escherichia coli* and *Bacillus subtilis*.

This paper describes the antitumor activity against various murine tumors, the acute toxicity, the bone marrow toxicity, and growth inhibitory activity on tumor cells *in vitro* and the macromolecule synthesis of HeLa S_3 cells.

Materials and Methods

Chemicals

Echinosporin was prepared according to the method of OKACHI *et al.*[1] Mitomycin C (Kyowa Hakko Kogyo Co., Ltd.) and vincristine (Sigma Chemicals, U.S.A.) were used as reference antitumor agents. [^{3}H]Thymidine ([*methyl*-^{3}H]thymidine 55 Ci/mmol), [^{3}H]uridine ([5-^{3}H]uridine 25 Ci/mmol) and [^{3}H]leucine (L-[4,5-^{3}H]leucine 156 Ci/mmol) were obtained from the Radiochemical Center Amersham (U.K.).

Antitumor Activity

Antitumor activity against leukemia P388, sarcoma 180 and melanoma B16 was studied as described in the previous report.[3] Fibrosarcoma Meth 1 which had originally been induced by 3-methylcholanthrene in BALB/c mice in our laboratory has been maintained in ascites form in BALB/C mice.[4]

For the antitumor test, 1×10^6 cells of Meth 1 were inoculated ip into CDF_1 mice (20~22 g). Leukemia P388 resistant to vincristine (P 388/VCR) was kindly donated by Dr. M. INABA, Cancer Chemotherapy Center (Tokyo). P388/VCR cells (1×10^6/CDF mouse) were inoculated ip. Human xenograft MX-1 was donated by the Cancer Chemotherapy Center (Tokyo). Solid tumor (2^3 mm^3) was inoculated sc into BALB/c nu/nu mice by trocar and drugs were injected on day 14 when the tumor volume was about 125 mm^3. The tumor volume was calculated by the formula represented in the NCI protocol for screening.[5]

Bone Marrow Toxicity

For the peripheral white blood cell count, 20 μl of supraorbital venous blood was mixed with 9.98 ml of Cellkit-7 solution (Toa Medical Electro Co., Ltd.) and counted by microcell counter (Toa Medical Electro Co., Ltd.) after lysis of erythrocytes with saponin S (Toa Medical Electro Co., Ltd.). Differential counts of white blood cells were performed on Wright-stained smear of pretreatment blood

Table 1. Effect of echinosporin on leukemia P388.

	Dose (mg/kg/day)	Schedule[a]	MST (days)[b]	ILS (%)[c]
Control		day 1	9.4±0.5	—
Echinosporin	80	day 1	10.2±6.7	9
	60	day 1	15.4±1.3	64
	40	day 1	14.8±1.1	57
	20	day 1	12.6±0.9	47
	10	day 10	10.8±1.8	15
	30	day 1~5	7.8±1.3	0
	20	day 1~5	12.6±0.5	40
	10	day 1~5	12.4±0.9	38
	5	day 1~5	11.2±0.4	24
	2.5	day 1~5	9.4±0.5	4
Mitomycin C	6	day 1	26.2±7.4	179
	4	day 1	16.6±0.9	77
	1	day 1~5	22.0±3.9	144

[a] Leukemia P388 (1×10^6 cells) was inoculated ip in CDF_1 mice on day 0 and drugs were injected ip on day 1 or once a day for 5 days from day 1.
[b] Mean survival days ±SD.
[c] Increased life-span.
Each group consisted of 5 mice.

Table 2. Effect of echinosporin on P388 and P388/VCR.

	Dose (mg/kg)	ILS (%)[b]	
		P388[a]	P388/VCR[a]
Echinosporin	80	12	9
	60	64	45
	40	50	103
	20	33	23
	10	NT	19
Vincristine	4	−47	−2
	2	34	2
	1	41	−2
	0.5	41	5
	0.25	29	9

[a] Leukemia P388/VCR or P388 (1×10^6 cells) was inoculated ip on day 0 and echinosporin or vincristine was injected ip on day 1.
[b] Increased life-span.
Each group consisted of 5 mice.
NT: Not tested

on day 4 after treatment which was found to be the nadir of WBC depression.

In Vitro Studies

HeLa S_3 cells used in this study were kindly donated by Dr. K. KURODA, National Institute of Genetics (Mishima) and maintained in Eagle minimum essential medium (MEM) (Nissui Seiyaku Co., Ltd.) supplemented with 10% fetal bovine serum(FBS, Grand Island Biological Company, New York, U.S.A.) and glutamine (10 mM). HeLa S_3 cells (150 cells/5 ml) were plated in plastic dishes (9 cm diameter) (NUNC Co., Ltd., Denmark) and drugs were added 3 hours after plating. The treatment of cells with drugs was carried out by adding a small amount of phosphate buffer saline solution (PBS) (GIBCO, N.Y., U.S.A.) of the drug into the culture. After 30 minutes incubation with drugs, the cultures, including untreated control, were rinsed twice and then fed with fresh and prewarmed culture medium for further incubation. After 10 days, the number of colonies was counted and the average of 3 plates at each drug concentration was calculated. The plating efficiency of the untreated control culture varied from 80~90% in each experiment.

The precursor incorporation into the TCA insoluble fraction was measured by pulse-labeling of HeLa S_3 cells with [^{3}H]thymidine (0.5 μCi/ml), [^{3}H]uridine (0.5 μCi/ml) or [^{3}H]leucine (1.5 μCi/ml) at every hour from 60 minutes after adding the drugs at 37°C. Immediately after incubation with the radioactive precursors for 1 hour, the medium containing the radioactive precursor was removed by aspiration. The adherent cells were washed with 5% trichloroacetic acid (TCA) for 30 minutes and then with ethanol for 10 minutes. The dried cell residues were dissolved in 1 ml of 0.5 N NH_4OH and the

Table 3. Effect of echinosporin on fibrosarcoma Meth 1.

	Dose (mg/kg/day)	Schedule[a)]	MST (days)	ILS[b)] (%)	60-day[c)] survivors
Control			16.9± 3.0		
Echinosporin	80	day 1	2.6± 0.5	—	
	60	day 1	36.3± 7.8	118	
	40	day 1	38.0± 7	125	
	30	day 1	39.8±11.9	136	1/5
	20	day 1	36.2±14.0	114	1/5
	10	day 1	33.6±16.9	99	
	30	day 1~5	7.8± 1.8	0	
	20	day 1~5	47.2±11.1	179	1/5
	10	day 1~5	57.6± 5.4	241	4/5
	5	day 1~5	21.8± 7.8	29	
	2.5	day 1~5	23.8±10.2	41	
	1.25	day 1~5	25.6±13.2	51	
Mitomycin C	6	day 1	71.4±13.2	311	

a) Fibrosarcoma Meth 1 cells (10^3 cells/mouse) were inoculated ip on day 0 and drugs were injected ip on day 1.

b) ILS (%) were calculated including 60-day survivors and the survival times of 60-day survivors was defined as 60 days.

c) Each group consisted of 5 mice.

Table 4. Antitumor activity of echinosporin on murine experimental tumor system.

Tumor	System	Schedule	Optimal dose (mg/kg/day)	T/C×100	Day of evaluation
P388	ip-ip	day 1	60	164	
	ip-ip	day 1~5	20	140	
	iv-iv	day 1	60	117	
P388/VCR leukemia	ip-ip	day 1	40	203	
EL-4 leukemia	ip-ip	day 1	50	113	
Meth 1 fibrosarcoma	ip-ip	day 1	30	236	
	ip-ip	day 1~5	10	341	
B16 melanoma	ip-ip	day 1	50	132	
Sarcoma 180	sc-ip	day 1	60	85	day 7
	sc-iv	day 1	60	90	day 7
	sc-iv	day 1~5	50	46	day 7
Lewis lung carcinoma	sc-iv	day 1	20	57	day 10
MX-1	sc-ip	day 14~20	40	72	day 35

radioactivity of the aliquot was counted by a liquid scintillation counter.

KB cells which was kindly donated by Dr. S. TSUKAGOSHI, Cancer Chemotherapy Center (Tokyo) were cultured in MEM supplemented with 10% FBS and 10 mM glutamine. Cells (10^5 cells/ml) were plated in multidish 24 wells (NVNC) and the drugs in PBS solution were added on day 1. After incubation with drugs for 3 days, the culture media were discarded and washed with Ca^{++} free-PBS. Single cells were obtained by the incubation of 0.05% trypsin and 0.02 mM EDTA. The number of cells were counted by microcell counter.

Results and Discussion

Antitumor Activity

As shown in Table 1, echinosporin was effective against leukemia P388 implanted ip by a single injec-

Fig. 1. Effect of echinosporin on the peripheral white blood cell counts of normal *ddY* mice.

* $P<0.05$, ** $P<0.01$. (Student's t test)

●—● Echinosporin; 60 mg/kg ×1 iv, ▲—▲ echinosporin; 60 mg/kg ×1 ip, ●---● mitomycin C; 6 mg/kg ×1 iv.

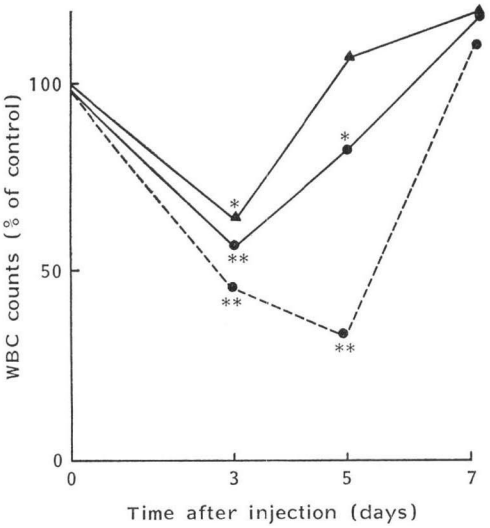


Fig. 2. Cell killing activity of echinosporin on HeLa S_3 cells.

D_0=9.8 μg/ml (n=4.7).

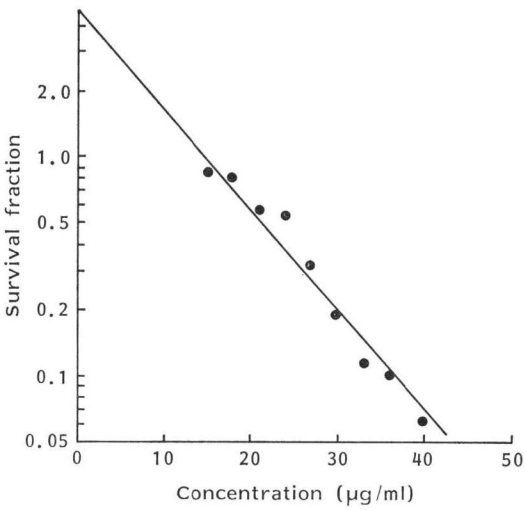
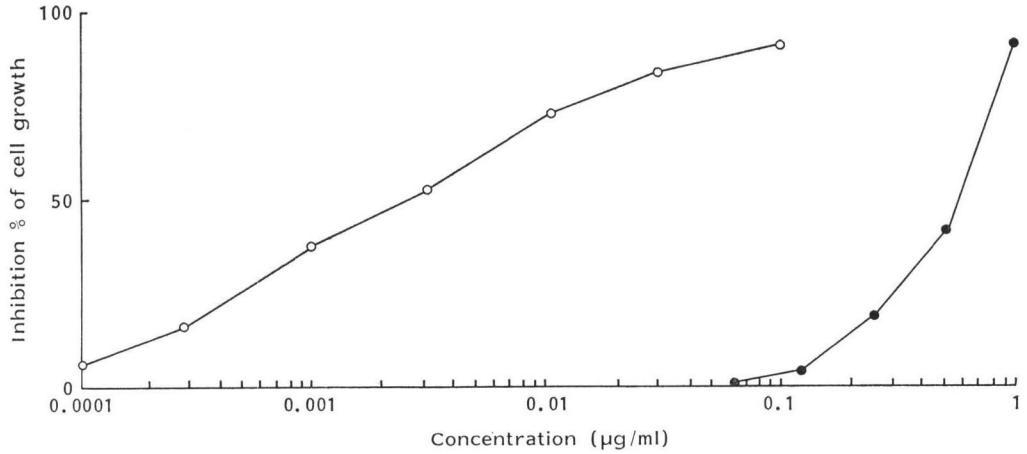


Fig. 3. Effect of echinosporin on the growth of KB cells.

KB cells (10^5 cells) were cultured for 24 hours at 37°C in MEM supplemented with calf serum. After addition of drugs, cells were incubated for another 72 hours. After decantation of culture fluid and washing, single cells were obtained by trypsin-EDTA and the numbers of cells were counted. Percentages of treated group to untreated group were calculated.

● Echinosporin, ○ mitomycin C.

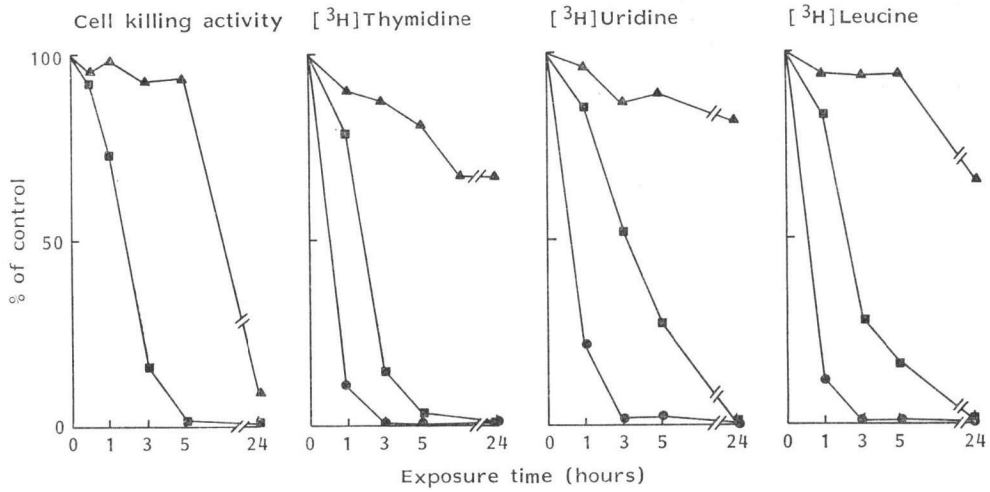


tion on day 1, or by successive administration once a day for 5 days. The optimal dose of a single administration was 60 mg/kg and gave ILS of 64%. With successive administration, it gave ILS of 40% at an optimal dose of 20 mg/kg/day. Significant activity was exhibited against mice bearing P388/VCR inoculated ip. The maximum ILS against P388/VCR was 103% at a dose of 40 mg/kg. It was more effective against P388/VCR than against P388 and showed the collateral sensitivity to P388/VCR

Fig. 4. Effect of echinosporin on colony formation of HeLa S_3 cells and macromolecule synthesis.

HeLa S_3 cells (200 cells) were cultured in the dish (5 cm diameter) in MEM supplemented with 10% FBS and treated with 1 (▲) or 5 (■) μg of echinosporin for the period shown in Fig. 4. Cells were washed with fresh medium and the numbers of colonies were counted after 10 days.

The radioactive precursors were added at the indicated time after addition of 1 (▲), 5 (■), 10 (●) μg of echinosporin and incubated for 1 hour. TCA were added and the radioactivity incorporated into TCA insoluble fraction was measured. % radioactivity of treated group to untreated control was determined.



(Table 2). Echinosporin gave a significant prolongation of the life-span against mice bearing Meth 1. It gave 80% of 60 day survivors by a consecutive 5 days administration at a dose of 10 mg/kg/day. The activity of echinosporin against Meth 1 was superior to that of mitomycin C (Table 3).

Echinosporin was only marginally active against ascitic tumor of B16 melanoma and gave 32% ILS at a single dose of 50 mg/kg. It was less effective than mitomycin C which gave 93% ILS with a single injection (Table 4).

Echinosporin was not active by a single ip administration on day 1 against the solid tumor of sarcoma 180. It was marginally active by iv and po 5 consecutive daily injections from day 1 and gave T/C of 0.46 and 0.41 respectively (Table 4). Echinosporin did not give a prolongation of the life-span of BDF_1 mice bearing EL-4 (10^6 cells/mouse, ip). For sc implanted Lewis lung carcinoma, it did not show the inhibition of the tumor growth or prolongation of life-span. The growth of MX-1 was not inhibited with T/C of 0.70 by a 7 consecutive iv administration (Table 4).

Bone Marrow Toxicity

At the ip and iv dose of echinosporin of 60 mg/kg, the WBC nadir occurred on day 3 post-treatment and was 63% and 56% of control, respectively. These values were not statistically significant from 56% of control WBC obtained with 6 mg/kg of mitomycin C (almost equal to dose of LD_{10}). But the WBC nadir occurred on day 5 after treatment with 33% of control at the iv dose of mitomycin C (Fig. 1). The depression of WBC count by echinosporin recovered more rapidly than that by mitomycin C. The WBC differential count revealed the damage to lymphocytes.

Effect on Cells *In Vitro*

Cell killing activity of echinosporin was shown on HeLa S_3 cells. The cells exposed for 30 minutes

showed a dose-dependent exponential survival curve with a wide shoulder at low concentration range. The mean lethal dose (D_0, the dose to give 37% survival) was 9.8 μg/ml and n (extrapolating value of exponential portion of dose survival curve) was 4.7 (Fig. 2). This might indicate that the DNA damage induced by echinosporin could be repaired effectively from sublethal damage.

The growth of KB cells was inhibited *in vitro* by echinosporin. IC_{50} (concentration which gave 50% cell number of control) of echinosporin was 0.62 μg/ml at 72-hour exposure and was more than 200-fold higher than that of mitomycin C (Fig. 3). The dose dependent growth inhibition curve of echinosporin was different from that of mitomycin C. The ratio of IC_{90}/IC_{50} was 2 and more than 10 for echinosporin and mitomycin C, respectively.

Effect on Macromolecule Synthesis

Echinosporin inhibited [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]leucine incorporation into TCA insoluble fraction dose-dependently, [^{3}H]thymidine incorporation was relatively more significantly inhibited than the other two radioactive precursors incorporation at 5 μg/ml. But there was no significant difference in the inhibitory activity of echinosporin on the incorporation of them into TCA insoluble fraction (Fig. 4). DNA synthesis might be primarily inhibited, but further studies for the mechanism of action is needed.

The blood urea nitrogen value of serum of mice injected a single dose of 60 mg/kg (ip) did not deviate from that of control mice (unpublished data).

While the antitumor activity and spectrum of echinosporin were not superior to those of antitumor antibiotics which are now in clinical use, the uniqueness of the structure and the solubility are interesting and need further studies.

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