ANTITUMOR ACTIVITY OF A NEW ANTIBIOTIC, KAZUSAMYCIN

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The antitumor activity of a new antibiotic, kazusamycin, against various murine tumors was studied by various treatment schedules. Single, intermittent, and successive injections of the antibiotic were almost equally effective against Ehrlich carcinoma, IMC carcinoma and sarcoma 180 tumor, but successive injections showed more efficacy than the other schedules against Meth A fibrosarcoma and Lewis lung carcinoma. Interestingly, there was no clear dose response for the efficacy of kazusamycin on murine tumors. When HeLa cells were exposed to kazusamycin for 72 hours *in vitro*, the IC_{50} value was about 1 ng/ml, however the cytotoxicity of the antibiotic depended on the incubation time.

A new antitumor antibiotic, kazusamycin, was isolated from the culture broth of *Streptomyces* sp. No. 81-484 by UMEZAWA *et al.* in 1984[1]. Kazusamycin is active against some kinds of fungi, yeasts, and mammalian cells *in vitro*. The structure of this antibiotic has recently been determined[2], it consists of an unsaturated branched-chain fatty acid with a terminal δ-lactone. In the previous report, preliminary results of the antitumor activity of kazusamycin on sarcoma 180 tumor and P388 leukemia were described. The experiments reported here were designed to investigate the antitumor activity of kazusamycin on various murine tumors.

Materials and Methods

Animals

Male ICR, *dd*Y, CDF, BALB/c, and C_{57}BL/6, 6 weeks of age, were purchased from the Shizuoka Agricultural Cooperative Association (Hamamatsu).

Agents

Kazusamycin isolated in our laboratory was dissolved in small amount of dimethyl sulfoxide and Tween 80, and diluted with water (for *in vivo* examinations). [^{3}H]Thymidine ([^{3}H]TdR, 22 Ci/mmol), [^{3}H]uridine ([^{3}H]UR, 6.5 Ci/mmol), [^{3}H]leucine (60.0 Ci/mmol), and [^{3}H]acetic acid (4.7 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, UK.

Tumor Cell Lines

Lewis lung carcinoma and B16 melanoma were obtained from Dr. TSUKAGOSHI of the Cancer Chemotherapy Center (Tokyo) and were inoculated into C_{57}BL/6 mice for passage. IMC carcinoma, obtained from the Institute of Microbial Chemistry, and P388 leukemia were maintained in CDF_1 mice. Sarcoma 180 tumor obtained from the Sasaki Institute (Tokyo), and Meth A fibrosarcoma have been maintained in ICR and BALB/c mice, respectively.

Antitumor activity was evaluated by the increase in life span (ILS): $(T/C-1)\times 100\%$, where "T" is the mean survival days (MSD) of the treated group and "C" is the MSD of the control group. Surviving mice scored 61 days after implantation of tumors and mice remaining alive after this period of observation were considered cured. Cured mice were excluded from the calculation of ILS.

Cytotoxicity on HeLa Cells

HeLa S3 cells have been maintained in monolayers in Eagle's minimum essential medium (MEM)

Table 1. Effect of kazusamycin on Ehrlich carcinoma and sarcoma 180.

Treatment schedule	Total dose (mg/kg)	Ehrlich carcinoma			Sarcoma 180		
		MSD	Range	ILS (%)	MSD	Range	ILS (%)
Saline		20	15~26	0	11.4	9~14	0
Day 1	1.25	36.8	25~53	84 (1)	16.7	18~35	46
	0.63	41.8	25~54	109 (1)	25.6	21~32	124
	0.31	44.0	22~58	120 (1)	33.6	24~54	195
	0.16	43.0	34~58	115	23.0	15~28	102
	0.08	30.0	18~36	50 (2)	22.8	18~32	100
	0.04	48.4	31~60	142	23.8	12~36	109
	0.02	39.2	27~52	96	20.9	9~27	83
	0.01	31.8	21~43	59 (1)	18.6	14~25	63
Days 1, 5, 9	1.25	32.8	24~46	64	23.7	18~36	108
	0.63	37.3	22~46	87 (2)	25.8	21~36	126
	0.31	35.5	22~45	78 (1)	30.2	20~57	165
	0.16	39.0	36~45	95 (2)	33.0	21~46	189
	0.08	39.5	34~42	98 (1)	36.4	22~49	219
	0.04	40.0	30~51	100 (1)	25.3	14~47	122
	0.02	27.0	20~34	35 (1)	24.1	8~35	111
	0.01	15.4	12~18	−23	18.6	13~32	63
Days 1~5	0.31	7.3	7~ 8	−64 (1)	14.0	4~50	23
	0.16	43.3	36~55	117 (2)	11.8	5~21	4
	0.08	46.0	43~48	130 (1)	30.0	21~42	163
	0.04	42.0	33~47	110 (2)	19.2	13~28	68 (1)
	0.02	23.4	12~49	17	22.4	8~42	96 (2)
	0.01	19.0	15~29	−5	19.6	8~39	72

Ehrlich carcinoma cells (2.5×10^6) or Sarcoma 180 cells (1×10^6) were inoculated ip into *dd*Y or ICR mice female, 6 weeks old, respectively. Mice were given ip injections of the antibiotic. Numbers in parenthesis indicate 61-day survivors out of 5 treated mice.

Survivors were excluded from the range and the calculation of ILS.

supplemented with 10% bovine serum and kanamycin (100 μg/ml) at 37°C. To determine the cytotoxicity of kazusamycin, HeLa S3 cells (4×10^4) in 2 ml of medium were placed in a tissue culture plate (Falcon, 24-Well) and incubated for 48 hours at 37°C in a 5% CO_2 - 95% air atmosphere. Each culture well was refed with fresh medium containing a different concentration of kazusamycin, and reincubated. HeLa cells were counted periodically for a further 72 hours. To determine the effect of pulse treatment of kazusamycin on HeLa cells, 48-hour culture cells were exposed to the antibiotic for 3 hours, and were rinsed 3 times with fresh MEM. The cells were then reinoculated for 72 hours, and the cells were counted.

Measurement of Macromolecular Synthesis

The cells (5×10^4 cells/0.2 ml) were plated on a cover slip (LUX, 13 mm Round) inserted previously into a tissue culture plate (Falcon, 24-Well), and 2 ml of MEM was added after 3 hours. Following 48 hours of incubation, each culture well was refed with fresh medium containing a different concentration of kazusamycin. HeLa cells were exposed to kazusamycin for 1~8 hours. One hour before termination of culture, various precursors were added. At the end of the incubation period, cells were washed with ice-cold phosphate buffered saline, and three times with ice-cold 5% trichloroacetic acid. The radioactivity of acid-precipitable material on the cover slip was measured by a Aloka liquid scintillation spectrometer.

Results

Antitumor Activity of Kazusamycin

As shown in Tables 1 and 2, kazusamycin was effective against Ehrlich carcinoma, sarcoma 180

Table 2. Effect of kazusamycin on IMC carcinoma.

Treatment schedule	Total dose (mg/kg)	MSD	Range	ILS (%)
Saline		14.6	13~16	0
Day 1	0.63	11	3~22	−25
	0.31	30.8	18~52	111
	0.16	40.7	20~54	179 (2)
	0.08	47.8	43~50	227 (1)
	0.04	28.5	19~42	95 (1)
	0.02	30.7	18~54	110 (2)
Days 1, 5, 9	1.25	27.0	19~53	85 (1)
	0.63	43.6	24~52	199
	0.31	25.3	19~38	73 (2)
	0.16	32.8	18~50	125 (1)
	0.08	30.0	17~43	105 (3)
Days 1~5	0.16	19.8	6~29	36
	0.08	42.0	27~60	188 (1)
	0.04	45.6	39~49	212
	0.02	53.0	48~58	263 (3)
	0.01	29.8	20~50	104

IMC carcinoma cells (1×10^6) were inoculated ip into CDF_1 mouse (female, 6 weeks old), and mice were given ip injections of the antibiotic.

Numbers in parenthesis indicate 61-day survivors out of 5 treated mice.

Table 3. Effect of kazusamycin on Meth A fibrosarcoma and P388 leukemia.

Treatment schedule	Total dose (mg/kg)	Meth A fibrosarcoma			P388 leukemia		
		MSD	Range	ILS (%)	MSD	Range	ILS (%)
Saline		13.2	11~16	0	12.3	11~14	0
Day 1	0.63	15.6	14~17	18	3.0	3	−76
	0.31	17.2	13~28	30	13.8	13~14	12.5
	0.16	14.6	13~16	11	13.2	13~15	17.9
	0.08	16.0	14~18	21	12.4	12~14	7
	0.04	14.8	14~16	12			
Days 1, 5, 9	1.25	18.8	15~25	42	18.0	16~20	46
	0.63	19.0	19	44	16.6	15~18	35
	0.31	19.4	19~21	47	15.0	13~18	22
	0.16	19.6	15~30	48	15.0	13~16	22
	0.08	18.4	14~29	39	15.2	13~18	24
	0.04	15.4	14~18	17	14.8	14~16	20
Days 1~5	0.31	7.8	4~17	−41 (1)	6.6	5~ 7	−46
	0.16	11.2	5~19	−15	7.3	7~ 8	−41
	0.08	18.0	17~19	36 (3)	16.0	12~18	30
	0.04	25.8	16~40	95	16.8	14~21	37
	0.02	15.2	13~16	15	14.6	13~16	19
	0.01	13.2	12~15	0	14.2	11~18	15

Meth A fibrosarcoma cells (1×10^6) or P388 leukemia cells (1×10^5) were inoculated ip into BALB/c or CDF_1 mice (female, 6 weeks old), respectively, and mice were given ip injections of the antibiotic.

Numbers in parenthesis indicate 61-day survivors out of 5 treated mice.

tumor and IMC carcinoma in all of the treatment schedules. In particular, one to three out of five mice bearing IMC carcinoma were cured at low doses of the antibiotic. Five consecutive treatments of Meth A fibrosarcoma were found to be considerably more effective than intermittent or a single treatment in increasing the life span of mice (Table 3). Tables 3 and 4 show the moderate effect of

Table 4. Effect of kazusamycin on B16 melanoma and Lewis lung carcinoma.

Treatment schedule	Total dose (mg/kg)	B16 melanoma			Lewis lung carcinoma		
		MSD	Range	ILS (%)	MSD	Range	ILS (%)
Saline		22.8	19~28	0	12.2	11~14	0
Day 1	0.63	21.6	20~33	−5	14.8	12~17	21
	0.31	23.6	20~26	3.5	15.4	13~17	26
	0.16	21.2	18~23	−7	13.2	12~15	8
Days 1, 5, 9	1.25	26.8	24~30	18	14.6	10~18	20
	0.63	29.0	24~31	27	15.6	14~17	28
	0.31	29.2	25~36	28	16.2	15~19	33
	0.16	28.4	24~36	25	15.8	12~18	30
	0.08	29.6	25~36	30	15.0	13~18	25
Days 1~5	0.16	27.2	24~29	19	12.2	6~24	0
	0.08	27.8	27~37	22	18.2	12~21	49
	0.04	27.2	24~35	19	18.4	13~21	51
	0.02	29.6	23~36	30	16.8	15~19	38

B16 melanoma or Lewis lung carcinoma were inoculated ip into C_{57} BL mice (female, 6 weeks old), and mice were given ip injections of the antibiotic.

Numbers in parenthesis indicate 61-day survivors out of 5 treated mice.

Table 5. Effect of kazusamycin on IMC solid tumor.

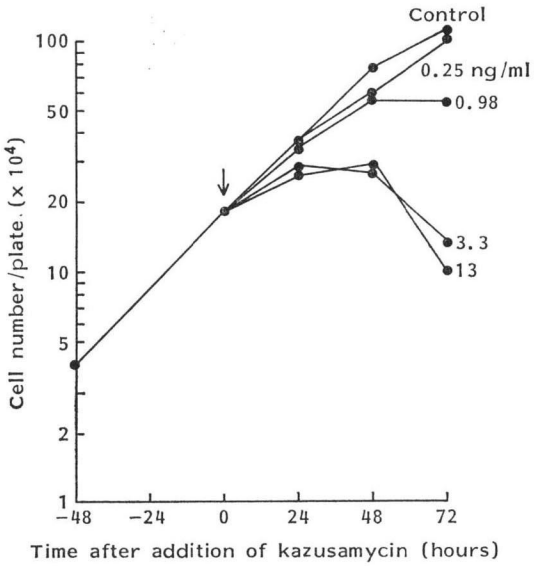
Total dose (mg/kg)	Mean tumor size (mm^2)		61-day survivors
	Day 29	Day 42	
Saline	312	1,193	0/5
0.16	169 (46)	658 (45)	2/5*
0.08	181 (42)	675 (43)	2/5*
0.04	120 (62)	439 (63)	2/5
0.02	215 (31)	618 (48)	0/5

IMC carcinoma (1×10^6) was inoculated subcutaneously into CDF_1 mouse (female, 6 weeks old), and mice were given ip injectors of the antibiotic on days 1~5.

Numbers in parenthesis indicate percent of growth inhibition. Control mice died between days 49~54.

* Without solid tumor.

Fig. 1. Cytocidal activity of kazusamycin on HeLa cells.



kazusamycin on Lewis lung carcinoma, whereas only slight effects were observed on P388 leukemia and B16 melanoma. Table 5 shows the antitumor activity of kazusamycin on IMC solid tumor. A moderate inhibition of tumor growth was noted at all doses, and some cured mice were observed at higher doses.

Cytotoxicity on HeLa Cells

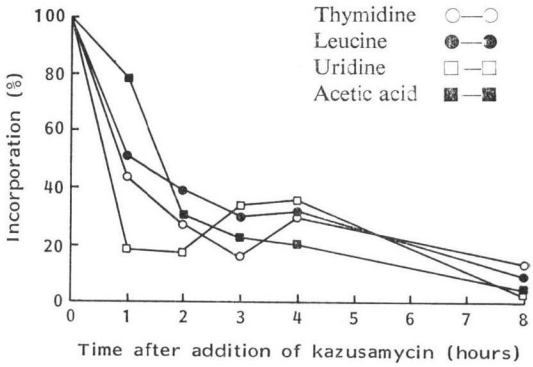
HeLa cells were exposed to various concentrations of kazusamycin and cell growth was determined by means of the cell count. As shown in Fig. 1, 0.98 ng/ml of kazusamycin inhibited the cell growth 72 hours after addition of the antibiotic. However, slight cell growth was observed in spite of higher doses of the antibiotic within 24 hours and thereafter the number of HeLa cells decreased. When

Table 6. Cytotoxicity of kazusamycin on HeLa cells (3 hours pulse treatment).

Concentration (ng/ml)	Inhibition of cell growth (%)
0	0
3.3	1.4
13.1	16.4
214	22.7
21,000	99.4

HeLa cells were exposed to kazusamycin for 3 hours, only slight inhibition was observed even at a concentration of 214 ng/ml (Table 6).

Fig. 2. Effect of 100 μg/ml kazusamycin on the synthesis of macromolecules in HeLa cells.



Effect of Kazusamycin on Macromolecular Synthesis

The effect of kazusamycin on the incorporation of various precursors into acid-precipitable macromolecules of HeLa cells was determined, periodically. When HeLa cells were exposed to 100 μg/ml of this antibiotic, the incorporation of these precursors was almost equally inhibited at 8 hours (Fig. 2), but 10 μg/ml or 1 μg/ml of the antibiotic did not show any inhibition (data not shown) although these concentrations were much higher than the IC_{50} value (1 ng/ml) observed when cells were exposed to kazusamycin for 72 hours (Fig. 1).

Discussion

It has been reported that kazusamycin showed unique antimicrobial activity and a remarkable cytotoxicity to HeLa cells[1]. In the present study, the antitumor efficacy of kazusamycin was determined by various treatment schedules using murine tumors. It was interesting that five successive injections were relatively effective at lower dose levels than a single injection although this schedule increased lethal toxicity of kazusamycin. When HeLa cells were exposed to kazusamycin for 72 hours, almost complete growth inhibition was observed even at a very low concentration (1 ng/ml) whereas 3 hours's exposure did not show severe cytotoxicity. In view of these findings, it is considered that kazusamycin is a time dependent drug according to the criteria of SHIMOYAMA and KIMURA[3].

Characteristically, with increasing dose of antitumor agent, the survival time of the animals increases progressively, reaches a peak, and then diminishes. Interestingly, there were no clear dose responses in the antitumor efficacy on sensitive tumor strains. For example, when sarcoma 180 was treated with intermittent injection of 1.25 or 0.02 mg/kg, the ILS was 108% and 111%, respectively. This indicates that the therapeutic index of kazusamycin is high.

In the inhibitory study of macromolecular synthesis in HeLa cells, all synthesis was inhibited at 100 μg/ml of the antibiotic but no particular inhibition was observed at 10 μg/ml. When HeLa cells were exposed to kazusamycin for 3 days, many polynuclear giant cells appeared at a concentration of 1 ng/ml (data not shown). Among the known antitumor agents, sporamycin, neocarzinostatin, mitomycin C and bleomycin produced the same type of morphological change[4,5]. An investigation of the mechanism of action is now in progress, and the results will be reported elsewhere.

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