

ANTITUMOR ACTIVITY OF SPERGUALIN, A NOVEL
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Spergualin (SGL), a novel antitumor antibiotic, exhibited strong antitumor activity against transplantable leukemias in mice: L1210, L1210(IMC), P388, P815, C1498, EL-4 and RL ♂1. It also exhibited antitumor activity against M5076 fibrosarcoma, AH66 and AH66F rat hepatomas, but not against Meth-A fibrosarcoma, B16 melanoma, Lewis lung carcinoma (LL) and C26 colon adenocarcinoma. The antitumor activity of SGL was administration-schedule dependent. The strongest activity against L1210 was obtained by ip continuous infusion for 7 days or daily ip administration for 9 days. Single ip injection of SGL at 100 mg/kg to mice caused convulsion and death within 15 minutes after injection. Such acute toxicity was not observed by continuous infusion. SGL showed its strongest activity at subtoxic dose against sensitive tumors except for L1210(IMC). Mice implanted ip with L1210(IMC) were cured by treatment with SGL at 3.13 mg/kg/day for 9 days, but died from the tumor at 50 mg/kg/day × 9. The cured mice rejected a second inoculation of up to 10⁶ tumor cells. The tumor cells isolated from mice after treatment with the high dose showed resistance to SGL *in vivo*. Mice implanted sc with L1210(IMC) were also cured by 9 daily ip administrations of SGL at 12.5 mg/kg/day, but solid tumor was observed at the implantation site until 3 days after the final injection of SGL in some cured mice. These results suggest that the therapeutic effect of SGL has a relatively high specificity for leukemias and that the immunological effect is involved in the antitumor activity.

Spergualin (SGL), a novel antitumor antibiotic, is produced by a strain of *Bacillus laterosporus*, and its structure was determined to be (–)-(15*S*)-1-amino-19-guanidino-11,15-dihydroxy-4,9,12-triazanonadecane-10,13-dione.¹⁾ SGL exhibits antitumor activity against transplantable murine tumors such as L1210 leukemia, EL-4 leukemia, Ehrlich carcinoma and sarcoma 180.²⁾ Moreover, a possible involvement of immune-enhancement by low doses of SGL and immuno-suppression by high doses, in the antitumor activity of SGL against L1210(IMC) has been suggested.³⁾

In this paper, we will report on the antitumor activity of SGL against a wide variety of experimental tumors and the effect of administration schedules on the antitumor activity against L1210 leukemia. We will also report on the involvement of the immunological effect on the antitumor activity of SGL and on the induction of resistant cells of L1210(IMC) cells.

Materials and MethodsSpergualin Trihydrochloride

SGL was prepared by Takara Shuzo Co., Ltd. (Ohtsu, Japan), and dissolved in physiological saline and stored in the dark at 4°C before use.

Table 1. Evaluation systems for antitumor activity of SGL.^a

Tumor	Origin	Host	Site	Size	Parameter	Period ^e
L1210	Lymphoid leukemia	CDF ₁ ♀	ip, sc	1 × 10 ⁵	MeST ^b	30
L1210(IMC)	Lymphoid leukemia	CDF ₁ ♀	ip, sc	1 × 10 ⁵	MeST, MTV ^c	30
P388	Lymphocytic leukemia	CDF ₁ ♀	ip	1 × 10 ⁶	MdST ^d	30
P815	Mastocytoma	DBA/2 ♀	ip	1 × 10 ⁶	MeST	30
C1498	Myeloid leukemia	C57BL/6 ♂	ip	1 × 10 ⁶	MeST	60
EL-4	Thymoma	C57BL/6 ♂	ip	1 × 10 ⁶	MeST	30
RL ♂ 1	X-Ray induced leukemia	BALB/c ♂	ip	1 × 10 ⁶	MeST	30
AH66	Hepatoma	Donryu ♀	ip	1 × 10 ⁶	MeST	60
AH66F	Hepatoma	Donryu ♀	ip	1 × 10 ⁶	MeST	60
M5076	Fibrosarcoma	C57BL/6 ♀	ip	2 × 10 ⁵	MdST	60
Meth-A	Fibrosarcoma	BALB/c ♀	ip	2.5 × 10 ⁵	MeST	60
B16	Melanotic melanoma	BDF ₁ ♂	ip	1 × 10 ⁵	MdST	60
LL	Lung carcinoma	BDF ₁ ♂	iv	1 × 10 ⁵	MdST	60
C26	Colon adenocarcinoma	CDF ₁ ♂	ip	5 × 10 ⁵	MdST	60

^a SGL was administered ip daily for 9 days.

^b MeST, mean survival time.

^c MTV, mean tumor volume.

^d MdST, median survival time.

^e Days.

Animals

DBA/2, C57BL/6, BALB/c (Charles River Japan Corp., Atsugi, Japan), CDF₁ (BALB/c × DBA/2), BDF₁ (C57BL/6 × DBA/2) and ICR mice (Shizuoka Laboratory Animal Center, Hamamatsu, Japan) and Donryu rats (Nippon Rat Co., Urawa, Japan) were used in experiments at an age of 6 to 7 weeks. Each dosing group was composed of at least 5 animals.

Tumors

To evaluate the antitumor activity of SGL, 14 kinds of experimental tumors were employed. The host animals, sites and amounts of tumor cells used for inoculation are shown in Table 1. We used two strains of L1210. One (L1210) was supplied by the Cancer Chemotherapy Center (Tokyo, Japan), which has been maintained in an ascitic form in DBA/2 mice, and the other is its sub-strain, L1210(IMC), which has been maintained in CDF₁ mice at Institute of Microbial Chemistry (Tokyo, Japan). Other tumors have been maintained in ascitic or solid forms in our laboratories.

Antitumor Experiments

In order to obtain the antitumor spectrum of SGL against the 14 tumors shown in Table 1, SGL was injected ip once a day for 9 days from the day after tumor inoculation (day 1). SGL was injected in a volume of 0.1 ml/10 g or 0.2 ml/100 g body weight into mice or rats, respectively.

Schedule dependency of antitumor activity of SGL against L1210 was examined according to the modified Type I protocol of National Cancer Institute (NCI, Bethesda, U.S.A.).⁴⁾ Treatment schedules examined were as follows: Single treatment (once); 6 times at intervals of 3 hours (q3h × 6); twice every 4 days (q4d × 2); 6 times a day every 4 days (q3h × 6-q4d × 3); 9 daily treatments (q1d × 9); continuous infusion for 7 days using an osmotic mini-pump Alzet 2001 model (Alza Corp., Palo Alto, U.S.A.). SGL was administered intraperitoneally from day 1. For continuous infusion, a mini-pump containing 200 μl of SGL solution was placed in the peritoneal cavity on day 1.

As shown in Table 1, antitumor activity was expressed by the T/C (%) value based on mean or median survival time, namely, (survival time in drug-treated group/that in control group) × 100. For sc inoculation of L1210(IMC), mean tumor volume was taken as a parameter as well as the mean survival time. Tumor volume (V) was calculated as follows:

$$V = L \times W^2 / 2,$$

where L and W are length and width of tumor, respectively.

Table 2. Antitumor effect of SGL against various transplantable tumors in mice and rats.^a

Tumor	Site	max T/C ^b	Optimal dose	min ED ^c	Index ^d
L1210	ip	222	50	1.4	71
	sc	238	12.5	3.7	27
L1210(IMC)	ip	>448 (10/10) ^e	6.25	0.76	132
	sc	>345 (6/6)	12.5	3.3	30
P388	ip	183	50	2.2	45
P815	ip	202	50	4.0	25
C1498	ip	206	50	1.2	83
EL-4	ip	212	50	0.46	217
RL ♂ 1	ip	167	50	2.2	45
AH66	ip	170	100	45	4.4
AH66F	ip	224	50	1.6	125
M5076	ip	155	50	35	2.9
Meth-A	ip	104	25	—	—
B16	ip	97	6.25	—	—
LL	iv	115	50	—	—
C26	ip	94	50	—	—

^a SGL was administered ip daily for 9 days.

^b max T/C: Maximum value of T/C (%).

^c min ED: Minimum effective dose, which means dose affording 150% of T/C (mg/kg/day × 9).

^d Index = (Lethal dose)/(Minimum effective dose).

^e Number in parentheses shows survivors 30 days after inoculation.

Results

Antitumor Spectrum of SGL

The antitumor activity of SGL against 14 experimental tumors is summarized in Table 2. SGL was effective in inhibiting all 7 leukemias transplanted intraperitoneally in mice. It was also effective against L1210 and L1210(IMC) transplanted subcutaneously. Among other non-leukemic tumors, SGL exhibited activity against M5076 fibrosarcoma, AH66 and AH66F rat hepatomas, but not against Meth-A fibrosarcoma, B16 melanoma, Lewis lung carcinoma (LL) and C26 colon adenocarcinoma.

SGL exhibited an antitumor effect over a wide dose range against SGL-sensitive tumors except against M5076 fibrosarcoma and AH66 rat hepatoma (see index in Table 2). SGL showed its strongest antitumor effect against L1210(IMC); almost all mice treated with SGL at 3.13, 6.25 and 12.5 mg/kg/day survived during the observation period (Table 3). In the other 6 leukemias, M5076, AH66 and AH66F, maximum T/C's were around 200%, and all animals died during the observation period even at the optimal dose (50 or 100 mg/kg/day).

SGL also inhibited the growth of L1210(IMC) as a solid tumor implanted subcutaneously, with prolongation of life span. When treated with SGL at 12.5 mg/kg daily for 9 days from day 1, the mice became tumor-free by day 30, although 3 out of 6 mice had solid tumor on day 12. On the other hand, the growth inhibitory effect of 50 mg/kg/day was so strong that the solid tumor was not detected on day 12, but on day 16 in 3 out of 6 mice the solid tumor appeared and the mice died from the tumor during the observation period (Fig. 1).

Administration Schedule Dependency

The antitumor activity of SGL against L1210 was examined under various administration schedules. As shown in Table 4, continuous infusion using an osmotic mini-pump showed the best effect.

Table 3. Effect of SGL on survival time of mice inoculated ip with L1210(IMC).

Dose (mg/kg/day)	T/C (%)	Survival time (mean±SD)	Survivors on day 30
50.00	>352	>23.6±4.5	2/10
25.00	>401	>26.9±4.3	6/10
12.50	>437	>29.3±2.2	9/10
6.25	>448	>30.0±0.0	10/10
3.13	>416	>27.9±5.1	8/10
1.56	>216	>14.5±5.8	1/10
0.78	151	10.1±1.4	0/10
0.00	100	6.7±1.6	0/10

Table 4. Effect of administration schedules on antitumor activity of SGL against L1210.

Schedule	Dose (mg/kg)		T/C (%)
	Single	Total	
Once	100	100	Tox. ^a
	50	50	105
	12.5	12.5	102
	3.13	3.13	98
q3h×6	50	300	114
	12.5	75	107
	3.13	18.75	107
q4d×2	50	100	107
	12.5	25	107
	3.13	6.25	102
q3h×6 - q4d×3	50	900	129
	12.5	225	152
	3.13	56.25	136
q1d×9	50	450	206
	12.5	112.5	199
	3.13	28.13	163
	0.78	7.03	145
Alzet 2001	—	1,800	256
	—	450	210
	—	112.5	185

^a Mice died within 15 minutes after single administration of 100 mg/kg of SGL.

SGL caused only slight body weight loss when 50 mg/kg was injected daily for 9 days (450 mg/kg in total), but a single injection of 100 mg/kg caused convulsion and death within 15 minutes after the injection. Such lethal toxicity was not observed in the continuous infusion even at a total dose of 1,800 mg/kg.

Induction of Resistance to SGL

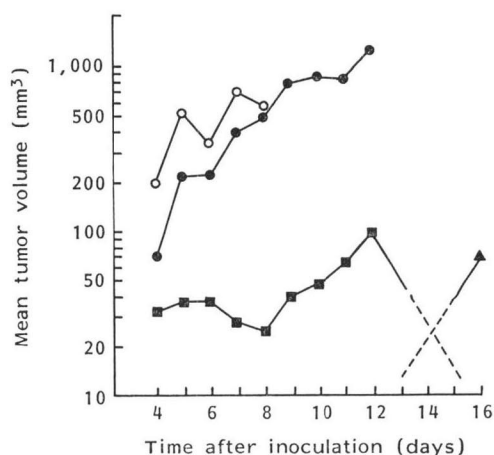
As shown in Table 3, the mice treated with a high dose (50 mg/kg/day) of SGL died from the tumor, whereas at the optimal dose (6.25 mg/kg/day) all mice were cured. We isolated the surviving L1210(IMC) cells from the peritoneal cavity after treatment with 50 mg/kg/day for 9 days and their

Fig. 1. Effect of SGL on the growth of L1210 (IMC) inoculated subcutaneously.

CDF₁ mice were inoculated sc with 10⁵ L1210 (IMC) cells and treated ip with SGL daily for 9 days. All mice in a control group (○) and SGL-treated group at 3.13 mg/kg/day (●) died from the tumor.

At 12.5 mg/kg/day (■), 3 out of 6 mice had the solid tumor on day 12, but the tumors were not detected on day 16 and all the 6 mice survived for 30 days without the solid tumor.

At 50 mg/kg/day (▲), no solid tumor was detected until day 12, but on day 16 the tumor appeared in 3 out of 6 mice and these 3 mice died from the tumor within 30 days. The remaining 3 mice survived for 30 days without detection of the solid tumor.



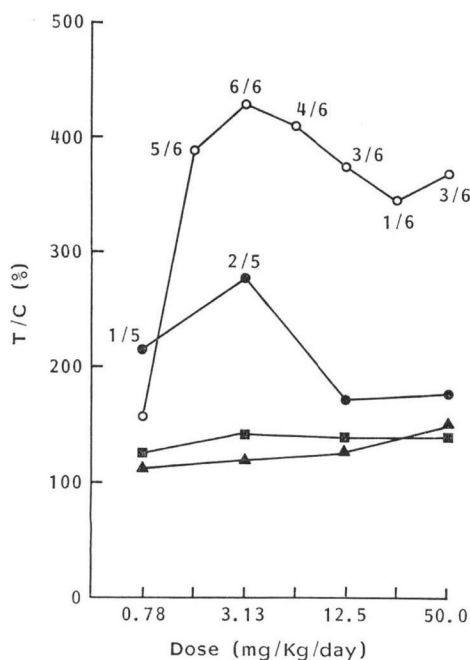
The activity shown by daily administration for 9 days (q1d×9) was comparable to that by continuous infusion at total doses of 112.5 and 450 mg/kg. In the treatment group of q3h×6 - q4d×3, the antitumor activity was much less than that by the above two schedules. In other schedules, once q3h×6 and q4d×2 administrations, SGL did not show any antitumor effect.

Fig. 2. Change of sensitivity of L1210(IMC) cells to SGL after treatment with SGL at 50 mg/kg/day for 9 days.

T/C value of SGL treatment of mice inoculated ip with intact L1210(IMC) cells (○) and of mice inoculated with surviving cells after 9 days treatment at 50 mg/kg/day (●).

This process was repeated twice (■) and 3 times (▲).

Numbers of survivors 30 days after inoculation are indicated near symbols.



(IMC) cells at various cell numbers. As shown in Table 5, the surviving mice rejected the second inoculation of L1210(IMC) up to an inoculum size of 10^8 cells.

Table 5. Survival time of mice after a second inoculation of L1210(IMC) cells at various cell numbers.

	Cell number	Survival time	Survivors on day 50
SGL-cured mice ^a	10^7	$>8.8 \pm 3.3^b$	1/5
	10^6	>50	14/14
	10^5	>50	13/14
Intact mice	10^7	5.2 ± 0.4^c	0/5
	10^6	6.0 ± 0.0	0/6
	10^5	7.0 ± 0.0	0/6

^a CDF₁ mice were ip re-inoculated with L1210 (IMC) cells 50 days after the first ip inoculation with 10^8 cells of L1210(IMC) followed by treatment with SGL at a dose of 5 mg/kg/day for 9 days.

^b Mean \pm SD excluding one survivor.

^c Mean \pm SD.

sensitivity to SGL was examined *in vivo*. As shown in Fig. 2, the surviving cells were resistant to SGL and at the optimal dose only 2 out of 5 mice survived. This re-transplantation of the surviving cells and treatment with SGL was repeated two further times, then the surviving cells became almost insensitive to SGL.

Rejection of Second Tumor Inoculation

Mice implanted ip with L1210(IMC) cells were treated with 5 mg/kg/day of SGL for 9 days, and the mice which survived 50 days after the implantation were re-inoculated with L1210

Discussion

In the present studies, it was found that all the 7 leukemias tested and 3 of the 7 non-leukemic tumors (M5076 fibrosarcoma, AH66 and AH66F rat hepatomas) were sensitive to SGL. The results suggested that SGL is specifically active against leukemias. This was supported by the result that 15-deoxyspergualin,⁵⁾ a SGL analog, exhibits a strong inhibition against *N*-butyl-*N*-nitrosourea-induced rat leukemias.⁶⁾

Studies on the effect of administration schedule of SGL on antitumor activity indicated that the anti-leukemic effect of SGL is dependent on the length of the period of exposure to SGL. Continuous infusion and daily administration for 9 days showed the strongest activity among the 6 schedules tested, and the continuous infusion showed the best result from the toxicological viewpoint.

SGL exhibited good activity against L1210(IMC) both in ip and sc inoculation systems. As reported previously,³⁾ the majority of ip implanted mice survived at low doses (3.13 ~ 12.5 mg/kg/day \times 9), and the surviving mice rejected a second ip inoculation of up to 10^8 L1210(IMC) cells. Moreover, the sc implanted mice became tumor-free by day 30 after administration of 12.5 mg/kg/day for 9 days,

although half of the mice had the solid tumor at the implantation site on day 12, namely, 3 days after the last injection of SGL. These results described above indicate that SGL has an immuno-modulating activity, and this activity contributes to the antitumor effect. L1210(IMC) cells isolated from mice after administration of 50 mg/kg daily for 9 days had become resistant to SGL. This may be one of reasons why L1210(IMC)-bearing mice treated with a high dose of SGL die from the leukemia, while the mice are cured by lower doses. As reported previously,³⁾ SGL shows the immuno-suppressive activity at high dose. This may be another reason for the death by leukemia by the high-dose treatment.

Further studies on the immunological involvement in the antitumor activity of SGL will be reported in a separate paper.

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