BIOLOGICAL ACTIVITY OF CADEGUOMYCIN

INHIBITION OF TUMOR GROWTH AND METASTASIS, IMMUNOSTIMULATION, AND POTENTIATION OF 1-β-D-ARABINOFURANOSYLCYTOSINE

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(Received for publication January 31, 1985)

Cadeguomycin retarded growth of sc solid IMC carcinoma in CDF_1 mice, and pulmonary metastasis of Lewis lung carcinoma in C57BL/6 mice. The antibiotic enhanced phagocytic activity of murine peritoneal macrophages and IL-1 production by $P388D_1$ cells. Delayed type hypersensitivity was stimulated and interferon was induced by the drug. The results suggest that cadeguomycin inhibits tumor growth and metastasis in association with modification of the immune system.

The cytotoxicity of arabinosylcytosine to K562 and YAC-1 cells was markedly enhanced by cadeguomycin in culture. The combined administration of arabinosylcytosine and cadeguomycin displayed potentiation in the inhibition of growth of ip-implanted P388 leukemia and metastasis of sc-implanted P388 leukemia to the regional lymph nodes.

Cadeguomycin showed low toxicity for mice.

We have isolated cadeguomycin, 7-carboxy-7-deazaguanosine, from a strain of *Streptomyces hygroscopicus*. The production, purification and structure assignment of the antibiotic have been described in the previous papers[1,2]. Cadeguomycin displays an unique property of enhancing uptake of pyrimidine nucleosides into K562 and YAC-1 cells[2,3]. We have studied the biological activity, because of this unique nature, and here present the results.

Materials and Methods

Chemicals

Cadeguomycin was prepared from culture broth of *Streptomyces hygroscopicus* IM7912T by Meiji Seika Kaisha, Ltd., following the method previously described[1,2]. The 1 mg/ml solution of cadeguomycin used was demonstrated to be endotoxin negative utilizing *Limulus* test (<0.1 ng/ml) (Wako Pure Chem. Ind.) 1-β-D-Arabinofuranosylcytosine was a product of Yamasa Shoyu Co., Ltd.

Animals and Tumors

Female ICR, C57BL/6 and CDF_1 mice, propagated at Charles River Japan, Atsugi, Kanagawaken, were used. IMC carcinoma is an ascitic tumor spontaneously induced in a female CDF_1 mouse (15 weeks old)[4]. IMC carcinoma and P388 leukemia were maintained in ascitic form in CDF_1 mice, and Lewis lung carcinoma as sc solid tumor in C57BL/6 mice.

Metastasis of P388 Leukemia in the Regional Lymph Nodes

A suspension of 3×10^5 P388 cells was implanted sc in the right fore-footpad of female CDF_1 mice (7~8 weeks old). On day 12, the number of tumor cells in the right axillary lymph nodes was assayed by implanting the lymph nodes ip into 10 CDF_1 mice and observing the survival period. The number of tumor cells was estimated by calibration curve.

Cells

K562 cell line was derived from human myelogenous leukemia[5], YAC-1 from murine lymphoma induced by Molony leukemic virus[6], and P388D_1, a macrophage cell line, from methylcholanthrene-induced murine lymphoid neoplasm[7,8]. The cells were grown in RPMI1640 medium containing 10% heat-inactivated fetal calf serum, benzylpenicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in atmosphere of 5% CO_2 and 95% air.

Delayed-type Hypersensitivity (DTH)

The DTH response to sheep red blood cells (SRBC) was assayed by the method of LAGRANGE *et al.*[9]. Female ICR mice (8 weeks old) were immunized by injection of 10^8 SRBC in 0.05 ml of saline into the right hind-footpad (sc). Four days later, 10^8 SRBC were injected sc into the left hind-footpad, and after 24 hours the animals were examined for increase in footpad thickness.

Phagocytosis of Yeasts by Macrophages

Female CDF_1 mice (8 weeks old) were given an ip injection of 1.0 ml of thioglycollate broth (Eiken Chemical Co.). After 4 days, exudate cells were collected by washing the peritoneal cavity of the animals with 5~6 ml of DULBECCO's phosphate-buffered saline (DPBS) (GIBCO). The cells were further washed and suspended in EAGLE's minimal essential medium (MEM) at 5×10^5/ml. The cell suspension (1.0 ml each) was placed in 3 plastic dishes (35-mm diameter, Falcon 3001), and incubated at 37°C for 2 hours in an atmosphere of 5% CO_2 and 95% air. Non-adherent cells were removed by washing the dishes with DPBS. Cadeguomycin solution in 1.0 ml of MEM was added to the macrophage monolayer, and incubated at 37°C for 60 minutes. The dishes were washed with DPBS, and 1.0 ml of DPBS with 0.2% bovine serum albumin and 0.2% glucose, containing 7.5×10^6 cells of heat-inactivated (100°C for 1 hour) *Saccharomyces cerevisiae*, was added to the macrophage layer. After 45-minute incubation at 37°C in 5% CO_2 and 95% air, the dishes were washed 3 times with DPBS, and the cells were fixed in methanol and stained with May-Grünwald and Giemsa solution, and the number of phagocytic cells was determined. Usually 80~110 phagocytic cells were observed out of 400 macrophages in control experiments.

Interferon (IFN) Induction

Female CDF_1 mice (8 weeks old) were injected ip with 1, 4 or 16 mg of cadeguomycin/kg and the sera were collected after 18, 24 or 30 hours. Each group consisted of 3 mice. The IFN level was assayed by the cytopathic effect (CPE) of vesicular stomatitis virus (VSV) in a 96-well microplate (Falcon 3072). Each well contained 0.1 ml of 2-fold-diluted serum and 0.1 ml of 7×10^4 L-929 cells in MEM with 10% calf serum. The microplate was kept at 37°C for 30 minutes in 5% CO_2 and 95% air. VSV of 100 ID_{50} in 0.1 ml of MEM with 1% calf serum was added to the cells, after washing with the medium. The infected cells were further incubated for 20 hours, in which controls reached the peak of CPE. The reciprocal of serum dilution, which showed 50% inhibition of CPE, was taken as IFN potency. The potency was corrected for international unit by comparison with reference IFN: NIH (L-929-NDV).

Interleukin (IL)-1 Production

IL-1 assay was carried out by [^{3}H]thymidine incorporation into murine thymocytes, enhanced by the supernatant of P338D_1 cell culture. P388D_1 cells (2×10^6/dish) were cultured in RPMI1640 medium with 1% fetal calf serum in the presence of cadeguomycin for 72 hours at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The 1/4-diluted culture supernatant (0.1 ml) was added to 0.1 ml of peanut agglutinin-free fraction of thymocytes of female C3H/He mice (5 weeks old). The thymocytes were cultured in the presence or absence of phytohemagglutinin (1 μg/ml) for 48 hours at 37°C in a 96-well microplate, and then [^{3}H]thymidine (0.2 μCi/well) (25 Ci/mmol, Amersham Japan) was incorporated into the cells for 18 hours.

Potentiation of Cytotoxicity of 1-β-D-Arabinofuranosylcytosine (Ara C)

K562 cells (2×10^4/ml) were incubated with cadeguomycin and Ara C for 72 hours at 37°C, and viable cell number was measured by trypan blue dye exclusion.

Results

The Effect on Growth of sc Solid IMC Carcinoma

Cadeguomycin was found to prevent growth of sc solid tumor of IMC carcinoma in CDF_1 mice. Approximately 50% inhibition was observed, when 4 or 8 mg of cadeguomycin/kg/day was ip injected for 8 days from day 11 to day 18 and tumor weights were assayed on day 30. The results are presented in Table 1. A similar effect was demonstrated by the treatment from day 1 to day 9 (data are not shown).

The Effect on Pulmonary Metastasis of Lewis Lung Carcinoma

Cadeguomycin retarded pulmonary metastasis of Lewis lung carcinoma (3LL) in C57BL/6 mice, although the primary tumor was not significantly affected (Table 2). On day 20, the number of metastatic foci was markedly reduced by ip administration of 4~16 mg of cadeguomycin/kg/day for 7 days. Approximately 50% inhibition was observed by the treatment from day 1 to day 7, and a similar effect from day 7 to day 13.

The Effect on Phagocytic Activity of Macrophages

The *in vitro* effect of cadeguomycin on peritoneal macrophages was studied by the method described in Materials and Methods. As illustrated in Fig. 1, cadeguomycin significantly stimulated the phagocytic activity of macrophages. Approximately 40% enhancement was induced at an antibiotic concentration of 0.4 μg/ml ($P<0.01$), and higher concentrations showed less effect.

Table 1. Effect of cadeguomycin on growth of sc solid IMC carcinoma.

Dose (mg/kg/day)	Schedule	Tumor weight (g)	% Inhibition
—		5.88±2.25	
16	Day 11~18	4.12±1.66	30
8		2.83±1.48	52 ($P<0.01$)
4		3.01±1.25	49 ($P<0.01$)

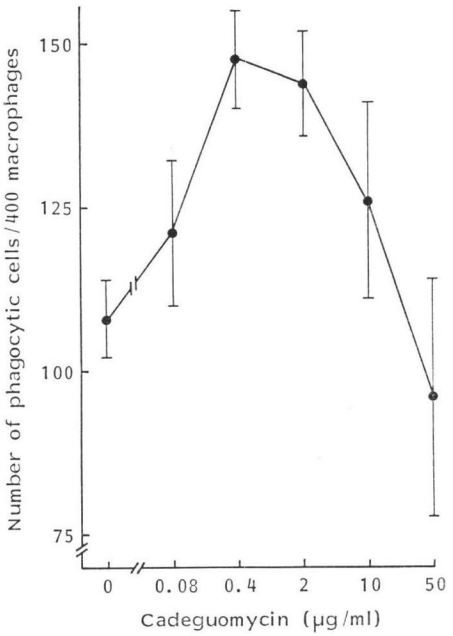
Each group consisted of 10 mice with 20 control animals. A suspension of 10^6 cells was implanted sc in the inguinal region of female CDF_1 mice (9 weeks old). The animals were sacrificed and tumor masses were weighed on day 30. Cadeguomycin was injected ip once a day.

Table 2. Effect of cadeguomycin on pulmonary metastasis of Lewis lung carcinoma.

Dose (mg/kg/day)	Schedule	Tumor weight (g)	Lung weight (mg)	Metastatic foci	
				Number	% Inhibition
—		4.96±1.38	225±41	33.4±15.1	
16	Day 1~7	4.61±0.94	192±21	17.1± 8.2	49 ($P<0.01$)
8		5.19±1.41	214±52	15.3± 4.6	54 ($P<0.01$)
4		4.21±0.83	217±31	16.2± 9.8	51 ($P<0.01$)
16	Day 7~13	4.59±1.18	204±38	20.2±11.7	40 ($P<0.05$)
8		4.85±1.60	215±31	18.4±11.5	45 ($P<0.02$)
4		5.01±1.59	189±31	17.0±10.1	49 ($P<0.01$)

Each group consisted of 10 mice with 20 control animals. Female C57BL/6 (8 weeks old) were inoculated sc in the dorsal region with 1-mm^3 fragments of Lewis lung carcinoma (3LL). The weight of the primary tumor and the number of metastases were determined by autopsy on day 20. Cadeguomycin was given ip once a day.

Fig. 1. Effect of cadeguomycin on phagocytosis of yeasts by peritoneal macrophages (*in vitro*).



The Effect on Interleukin (IL)-1 Production

Cadeguomycin at a concentration of 0.1 μg/ml was demonstrated to enhance IL-1 formation by P388D$_1$ cells, a cell line of macrophage, in the presence of phytohemagglutinin. IL-1, produced by P388D$_1$ cells, stimulated [^{3}H]thymidine incorporation into murine thymocytes (Table 3).

The Effect on Delayed-type Hypersensitivity (DTH)

The effect on cell-mediated immunity was

Table 3. Effects of cadeguomycin on IL-1 production by P388D$_1$ cells.

Cadeguomycin (μg/ml)	[^{3}H]Thymidine incorporated (⊿dpm*)	
	Mitogen-free	with PHA**
0	7,188±1,046	7,498±1,050
0.1	8,248± 427	12,313±1,051 ($P<0.01$)
0.4	8,667±1,981	8,416±1,352
1.6	5,389±2,175	7,752±1,718
6.4	5,956±1,313	8,169±2,616
25.6	7,842± 959	8,260±1,847

IL (interleukin)-1 production was observed by [^{3}H]thymidine incorporation into murine thymocytes, enhanced by the supernatant of P388D$_1$ cell culture.

* ⊿dpm=(dpm of thymocytes with supernatant)−(dpm of thymocytes).

** PHA: Phytohemaggulutinin.

Fig. 2. Kinetics of interferon induction by cadeguomycin in mice.

○ 16 mg/kg, ● 4 mg/kg, △ 1 mg/kg.

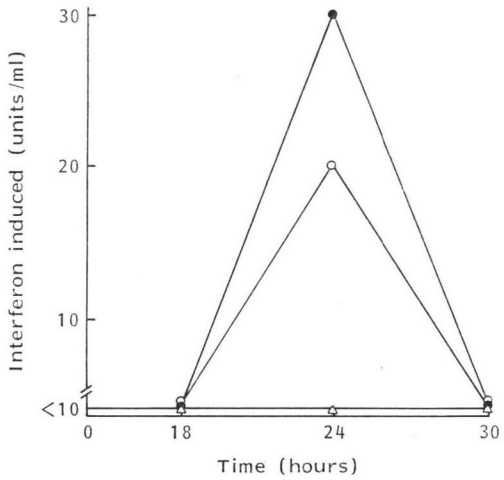


Table 4. Effects of cadeguomycin on DTH response to SRBC in mice.

Drug	Dose (mg/kg/day)	Schedule	Increase of footpad thickness (×0.1 mm)	T/C (%)
None			5.0±1.8	
Cadeguomycin	8	Day 1	5.4±1.5	108
	4		6.6±1.6	132
	2		5.9±2.3	118
Cadeguomycin	8	Days 1～3	8.6±3.1	172**
	4		7.6±3.6	152*
	2		4.2±1.6	84
Cadeguomycin	8	Day 4	7.3±2.3	146*
	4		7.3±2.4	146*
	2		4.5±2.1	90

* $P<0.05$, ** $P<0.01$.

Animal: Female ICR mice 8 weeks old. Each group consisted of 6 mice, and control 12 mice.

Fig. 3. Enhancement of arabinosylcytosine activity by cadeguomycin in K562 cells.

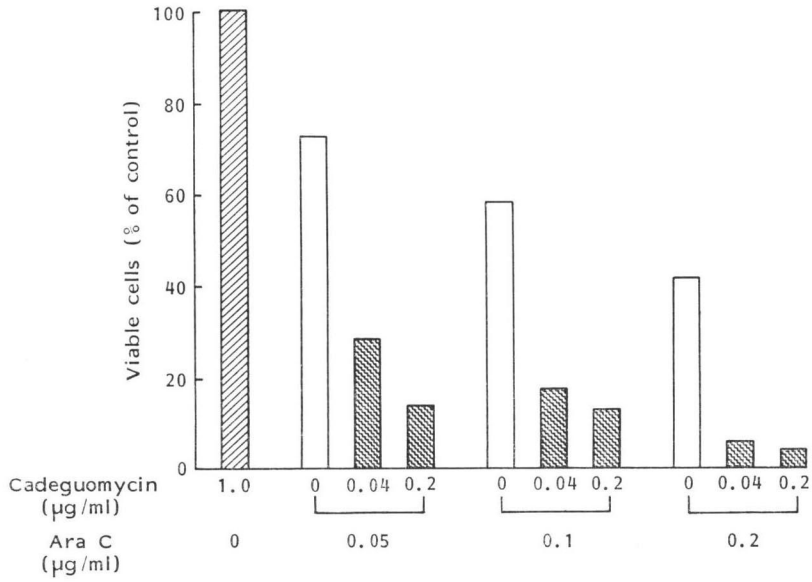


Table 5. Synergistic activity of cadeguomycin in combination with arabinosylcytosine on P388 leukemia.

Cadeguomycin (mg/kg/day)	Arabinosylcytosine (mg/kg/day)	MST (days)	T/C (%)
—		10.5±1.2	
8		10.8±1.3	103
4		10.3±0.8	98
2		10.7±1.5	102
—	100	14.3±0.5	136
8	100	15.2±0.8	145 ($P<0.05$)
4	100	15.5±0.8	148 ($P<0.05$)
2	100	14.8±1.0	141

Each group consisted of 6 mice. A suspension of 10^6 cells was implanted ip to female CDF_1 mice (7 weeks old). Cadeguomycin was injected ip for 10 days from day 1 to day 10, and arabinosylcytosine ip twice on day 1 and day 5.

studied by DTH response to SRBC in the footpad of ICR mice. As shown in Table 4, ip injection of 4 or 8 mg of cadeguomycin/kg/day for 3 days from day 1 to day 3 or once on day 4 significantly enhanced the DTH reaction.

The Interferon Induction

The effect of cadeguomycin on blood level of interferon was examined in CDF_1 mice. An ip injection of 4 or 16 mg of cadeguomycin/kg markedly induced interferon production, and high blood level of interferon was observed 24 hours after the antibiotic injection (Fig. 2).

The Potentiation of Cytotoxicity of 1-β-D-Arabinofuranosylcytosine (Ara C)

The cytotoxicity of Ara C to K562 cells was markedly enhanced by the presence of cadeguomycin (Fig. 3). For instance, 28% inhibition of cell growth was observed at an Ara C concentration of 0.05 μg/ml. The addition of cadeguomycin (0.04 μg/ml) led to 72% inhibition, and (0.2 μg/ml) to 86% inhibition.

Similar potentiation of Ara C cytotoxicity by cadeguomycin was observed with YAC-1 cells (data are not shown).

Cadeguomycin showed synergistic activity with Ara C on ip-implanted P388 leukemia in CDF_1 mice (Table 5). Ip administration of 100 mg of Ara C/kg/day on days 1 and 5 displayed an insignificant increase of life-span (ILS) (36%), whereas a significant ILS (45~48%) was observed in combination with ip treatment of 4 or 8 mg of cadeguomycin/kg/day for 10 days from day 1 to day 10.

Table 6. The synergistic effect of cadeguomycin with arabinosylcytosine on metastasis of P388 leukemia in the regional lymph nodes.

Cadeguomycin (mg/kg/day)	Ara C	Tumor cells in the lymph node ($\times 10^4$)	Bioassay of the lymph nodes		
			MST (days)	T/C (%)	Survivors
—	—	140	10.5±1.6		0/10
8	—	200	10.0±2.3	95	0/10
4	—	15	11.9±3.0	113	0/10
2	—	9.0	12.2±3.0	116	0/10
—	+	1.8	13.2±3.4	126*	0/10
8	+	0.14	14.9±3.5	142**	0/10
4	+	<0.01	>19.0±6.7	>181**	2/10*
2	+	0.6	13.9±4.5	132*	0/10

* $P<0.05$, ** $P<0.01$.

MST: mean survival time, Survivors: 30-day survivors/number of mice implanted with the lymph node.

The number of tumor cells in the axillary lymph node was estimated by calibration curve.

Each group consisted of 10 mice.

Cadeguomycin was injected ip for 10 days from day 1 to day 10, and Ara C (100 mg/kg) ip on days 1 and 5.

The combination of Ara C and cadeguomycin also exhibited potentiation in the inhibition of lymph node metastasis of sc-implanted P388 leukemia (Table 6). The combined ip administration of 4 mg of cadeguomycin/kg/day for 10 days from day 1 to day 10 and 100 mg of Ara C/kg/day on days 1 and 5 reduced the tumor cell number in the regional lymph nodes from 1.4×10^6 to less than 10^2, whereas cadeguomycin alone to 1.5×10^5, or Ara C alone to 1.8×10^4.

Acute Toxicity for Mice

Cadeguomycin displayed low toxicity for mice. Female ICR mice (5 weeks old) were not killed by an ip dose of 200 mg/kg nor by iv administration of 25 mg/kg. No loss of body weights nor gross lesions of any organs were observed. Higher doses were not studied, because of solubility of the antibiotic.

Discussion

The present study reveals that cadeguomycin inhibits growth of IMC carcinoma and metastasis of Lewis lung carcinoma, and enhances cell-mediated immunity and macrophage activity. An optimal dose for the effect is observed, suggesting that the antitumor activity is partly due to the immunomodifying effect. Since cadeguomycin stimulates IL-1 production, the enhancement of T cell-mediated DTH response may result from the macrophage activation.

Cadeguomycin exhibits an unique activity of stimulating the uptake of pyrimidine nucleosides into K562 and YAC-1 cells[2,3]. The current experiment displays potentiation of arabinosylcytosine by cadeguomycin for the *in vitro* cytotoxicity to K562 and YAC-1 cells, and for the *in vivo* inhibition of growth and lymph node metastasis of P388 leukemia. However, the mechanism of enhancement of nucleoside uptake and synergism with arabinosylcytosine remains to be determined.

Acknowledgments

This work was partly supported by a grant-in-aid for cancer research from the Ministry of Education, Science and Culture, Japan. The authors express their deep thanks to Dr. H. UMEZAWA, Institute of Microbial Chemistry, Tokyo, for his kind advice and cooperation in this study. They are greatly indebted to Meiji Seika Kaisha, Ltd. for the preparation of cadeguomycin.

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