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In vivo Antitumor Activity of Hexamethylmelamine against Human Breast, Stomach and Colon Carcinoma Xenografts

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We have evaluated the antitumor activity of Altretamine (hexamethylmelamine, HMM) on human carcinoma xenografts serially transplanted in nude mice. Five human breast carcinoma xenografts, MX-1, T-61, MCF-7, R-27 and Br-10, were inoculated subcutaneously into female nude mice. Two human stomach carcinoma xenografts, SC-1-NU and St-4, and three human colon carcinoma xenografts, Co-3, Co-4 and Co-6, were inoculated subcutaneously into male nude mice. One pellet of 17β-estradiol (0.1 mg/mouse) was inoculated subcutaneously in the mice transplanted with MCF-7 when the tumors were inoculated. HMM was administered per os daily for 4 weeks. MX-1 and T-61 tumors regressed completely after treatment with HMM at a dose of 75 mg/kg (the maximum tolerated dose: MTD) for MX-1 and 25 mg/kg for T-61. Br-10 was sensitive, whereas MCF-7 and R-27 were resistant to HMM at its MTD. HMM exerted the most potent antitumor effect against T-61. Against MX-1, it exerted an antitumor effect equivalent to that of cisplatin or cyclophosphamide. In addition, this agent was effective against all stomach and colon carcinoma xenografts, in particular St-4 (T/C% =10.7: the mean tumor weight of treated group/the mean tumor weight of control group) and Co-3 (T/C%=31.5%) which are insensitive to presently available agents. HMM seems worthy of further clinical investigation as a candidate agent to treat breast, stomach, colon and other carcinomas.

Key words: Altretamine — Hexamethylmelamine — Nude mouse — Chemotherapy

Altretamine (hexamethylmelamine, HMM) has been used for the treatment of ovarian, lung or breast carcinoma in Western countries (France, Germany, Italy, Israel, Denmark, Switzerland, Greece, Spain, Australia, Canada and the United States), and is regarded as the second-line treatment for these carcinomas, with a response rate of 20–30% in ovarian cancer refractory to primary cancer chemotherapy regimens, including cisplatin (CDDP).[1,2] The effect of HMM on breast cancer refractory to standard chemotherapy regimens has also been reported to be 20–30%.[3] The clinical investigation of the effect of HMM on colon, ovarian, uterine and lung cancers is at the phase II stage in Japan. Here, we report the antitumor activity of this agent on human carcinoma xenografts in nude mice administered HMM per os at various dose levels.

MATERIALS AND METHODS

Tumors Five human breast carcinoma xenografts (MX-1, T-61, MCF-7, R-27 and Br-10), two human stomach carcinoma xenografts (SC-1-NU and St-4) and three human colon carcinoma xenografts (Co-3, Co-4 and Co-6) were used for the experiments.[4]

These breast carcinoma xenografts are common ductal carcinomas which have been maintained by serial transfer in female nude mice. The gastric and colon carcinoma xenografts have been serially transplanted into male nude mice. MX-1 was established by Giovanella *et al.* from cancerous tissue of a 29-year-old female patient and was kindly supplied by Dr. K. Inoue, Cancer Chemotherapy Center, Tokyo. T-61 was derived from cancerous tissue of a 54-year-old female patient with breast cancer in Frankfurt am Main and was kindly supplied by Dr. N. Brünner, Copenhagen University. MCF-7 was established as a cultured cell line by Soule *et al.* in 1970. MCF-7 was first transplanted into nude mice treated with 17β-estradiol at Keio University in 1983. R-27 was established by Nawata *et al.* as a subclone of MCF-7 and cultured in medium containing tamoxifen; the dependency of R-27 on estradiol is reported to be lower than that of MCF-7. SC-1-NU was kindly supplied by the Department of Surgery, Nagoya University. The other xenografts were established in the Pathology Division, National Cancer Center Research Institute and Keio University.

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T-61, Br-10, R-27 and MCF-7 are positive for estrogen receptor (ER), but MX-1 is negative. One pellet containing 0.1 mg of 17β-estradiol, which is released within 3 weeks, was inoculated subcutaneously into the mice bearing MCF-7 on the same day the xenograft was transplanted.

Nude mice BALB/cA female nude mice were purchased from CLEA Japan, Inc., Tokyo. They were maintained under specific pathogen-free conditions in Isoracks at our experimental animal center and given sterile food and water. Six- to eight-week-old mice weighing 20–22 g were used for the experiment.

Tumor inoculations, measurement of tumors and evaluation of drug activity One fragment of tissue, approximately 3×3×3 mm in size, was transplanted using a trocar needle into the subcutaneous space on both sides of the backs of nude mice under ether anesthesia. Tumors were measured (length and width) with sliding calipers three times weekly by the same observer.

According to the method of Geran *et al.*,[5] the tumor weight in mg was calculated from the linear measurements using the formula: tumor weight (mg) = length (mm) × (width in mm)^2/2. The administration of HMM per os was initiated 2 or 3 weeks after the tumor inoculation, when the estimated tumor weight had reached 100 mg. The effect of the agent on the tumor was expressed as the ratio of the treated to control group (T/C ratio). The antitumor activity was evaluated as positive when the lowest T/C ratio during the experiment was less than 42%, which is equal to $(0.75)^3$, reflecting a 25% reduction of each diameter. The toxicity of HMM was assessed based on the death rate and the maximum body weight loss of the mice during the experiments. To determine the maximum tolerated dose (MTD) of HMM, the dosage was increased from 12.5 mg/kg to 100 mg/kg.

Drug HMM (Altretamine; KB-913) was provided by Kanebo Ltd., Osaka. The powder of HMM was micronized and mixed with 1% aqueous solution of hydroxypropylcellulose using a mortar because of its water-insolubility. Mice were administered 12.5 mg and 25 mg of HMM per kg in the case of T-61, 37.5 mg and 75 mg of HMM per kg in the case of MX-1 and 75 mg of HMM per kg for the other xenografts, daily for 4 weeks except on Sundays. The agent, suspended in 0.2 ml vehicle per mouse, was given using a metallic gastric tube.

RESULTS

Breast carcinoma xenografts MX-1 and T-61 regressed completely after the treatment with HMM at a dose of 75 mg/kg and 25 mg/kg, respectively (Figs. 1 and 2). HMM suppressed the growth of MX-1 in a dose-

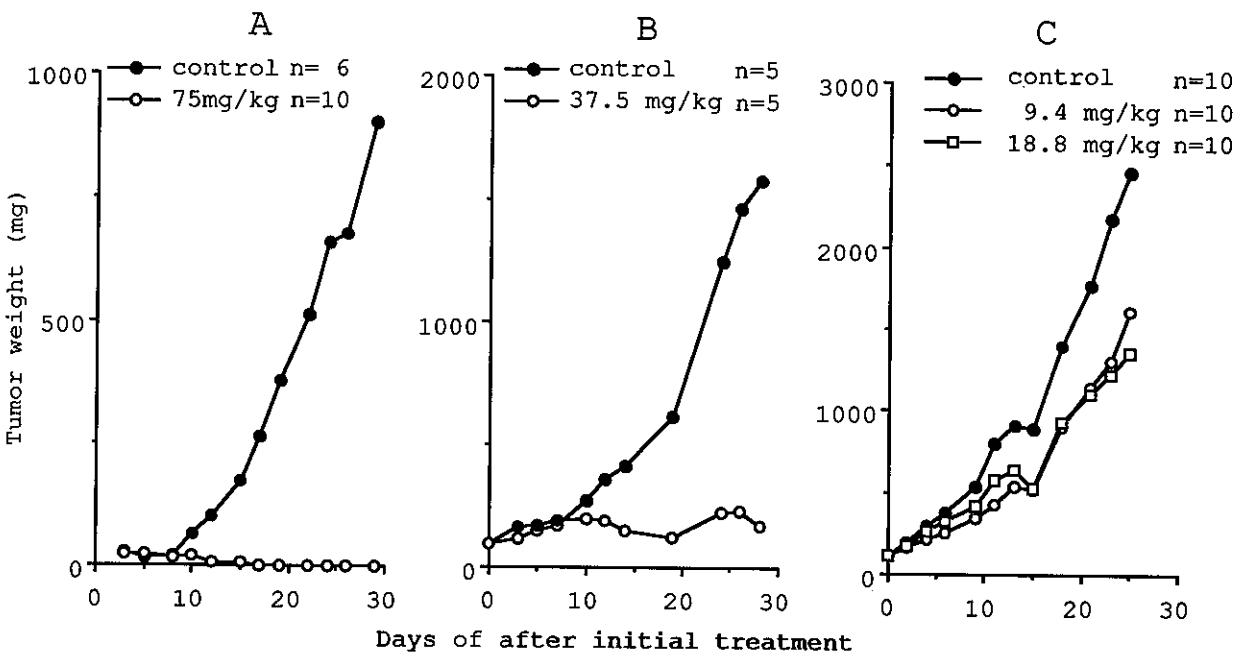

Fig. 1. Antitumor activity of hexamethylmelamine (HMM) on MX-1 xenografts in nude mice. Treatment was initiated when the mean tumor weight reached 100 mg. HMM was administered per os for 4 weeks every day except Sundays. The tumor growth curves of MTD (panel A), 1/2 MTD (panel B), 1/4 MTD and 1/8 MTD (panel C) of HMM-treated groups are shown. n: number of tumors.

dependent manner (Fig. 3) and this suppression was statistically significant (Student's t test) at doses of 9.4 (1/8 MTD) and 18.8 (1/4 MTD) mg/kg (Table I). In the case of T-61, the mean actual tumor weight regressed from 100 mg to 41.3 mg in mice administered 12.5 mg (1/6 MTD) of HMM. This effect was evaluated histologically as grade IIa, according to the criteria of the National Cancer Center,[6] that is, it showed cellular and structural destruction (Fig. 4). Br-10 was sensitive to HMM, but MCF-7 and R-27 were resistant to the drug. HMM showed more potent antitumor activity against

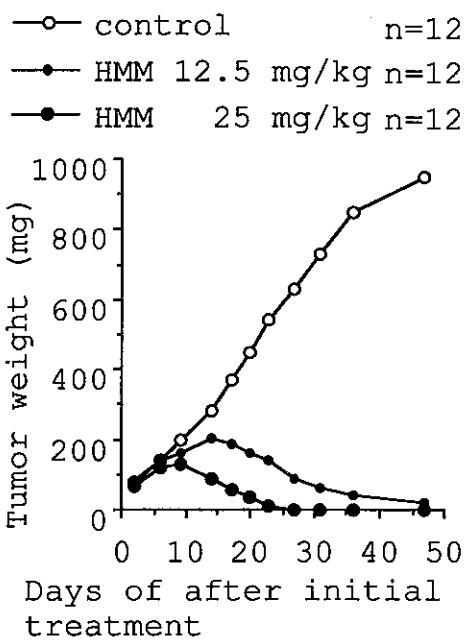

Fig. 2. Antitumor activity of hexamethylmelamine (HMM) on T-61 xenografts in nude mice. The treatment was initiated when the mean tumor weight reached 100 mg. HMM was administered per os for 4 weeks every day except Sundays.

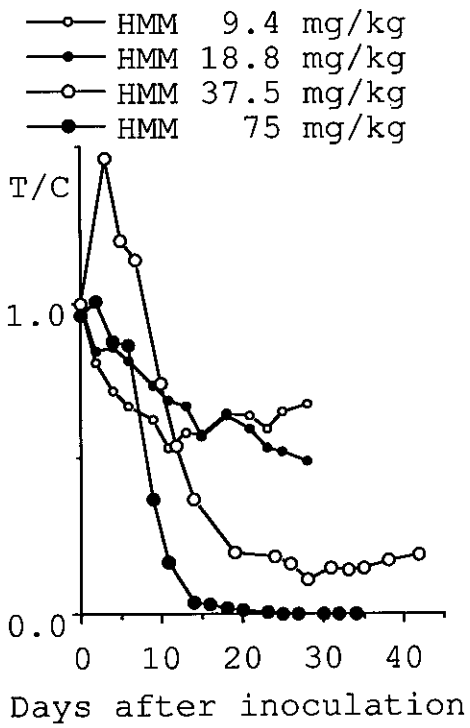

Fig. 3. Antitumor effect of hexamethylmelamine (HMM) on MX-1 xenografts in nude mice at various doses. Treatment was initiated when the mean tumor weight reached 100 mg. HMM was administered per os for 4 weeks every day except Sundays.

Table I. Antitumor Activity of Various Doses of Hexamethylmelamine on MX-1 and T-61: Human Breast Carcinoma Xenografts in Nude Mice

Xenograft	Treatment		Actual tumor wt. (mean±SD in mg)	T/C (%) of actual tumor wt.[a)]	Number of tumors
	Dose (mg/kg)	Schedule			
MX-1-110	control		2392.8±389.8		10
	9.4	qd×24 (1/8 MTD)	1406.9±809.6**	58.8	10
	18.8	qd×24 (1/4 MTD)	1074.8±616.0***	44.9	10
MX-1-103	control		1322.7±660.6		6
	75	qd×24 (MTD)	0	0	10
T-61-27	control		653.0±535.0		12
	12.5	qd×24 (1/6 MTD)	41.3±54.0*	6.4	12
	25	qd×24 (1/3 MTD)	0	0	12

Hexamethylmelamine was administered orally for 4 weeks every day except Sundays. The maximum tolerated dose of hexamethylmelamine is 75 mg/kg. The examinations were finished on day 28 (MX-1-110), day 34 (MX-1-103) or day 44 (T-61-27), when the nude mice were killed to measure the actual tumor weight.

a) The mean actual tumor weight of treated group/the mean actual tumor weight of control group.
* $P<0.05$, ** $P<0.01$ and *** $P<0.0001$ by Student's t test.

MX-1 and T-61 than the other drugs conventionally used for breast carcinomas[4] (Table II).

The minimum effective dose (MED) of HMM against MX-1 was 22.1 mg/kg, as calculated from the dose-dependence curve (Fig. 5). The chemotherapeutic index was 3.39 (MTD/MED).

Stomach and colon carcinoma xenografts HMM was effective against all stomach and colon carcinoma xenografts. The response rate with other representative anticancer agents varied from 0% to 50% (Table III). HMM was especially effective on St-4, which was resistant to all the other agents; it regressed to 51.8 mg on day 28 of treatment with HMM (Fig. 6).

Toxicity No deaths due to drug-induced toxicity were encountered up to a dose of 75 mg/kg at a schedule of qd×24 and no more than 20% body weight loss was observed in treated mice under the experimental conditions of this study. When HMM was administered at a daily dose of 100 mg/kg, one of five mice died on day 22 with more than 20% of body weight loss (Table IV). Based on these results it was considered that the MTD of HMM is 75 mg/kg q4d×24.

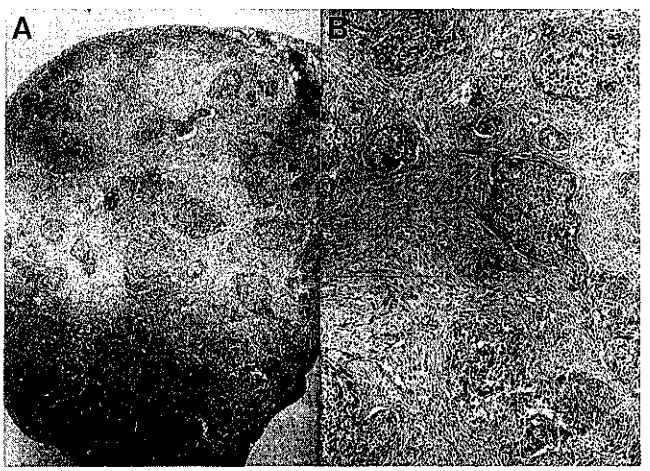

Fig. 4. Histological findings of antitumor effect of hexamethylmelamine (HMM) on a human cancer xenograft. In the case of T-61, the mean actual tumor weight regressed to 41.3 mg on treatment with 12.5 mg (1/6 MTD) of HMM, and this effect was evaluated histologically as grade IIa, showing cellular and structural destruction according to the criteria of the National Cancer Center. (HE stain. Magnifications, A: ×100, B: ×250)

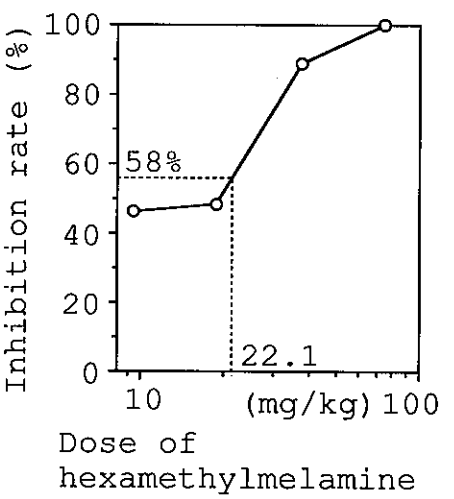

Fig. 5. Antitumor activity of hexamethylmelamine on MX-1 at different doses. The drug was evaluated as effective when the inhibition rate was more than 58%. Inhibition rate = (100 − minimum T/C%). Minimum T/C was the lowest ratio of mean tumor weight of the treated group to the mean tumor weight of control group during the experiment.

Table II. Effect of Drugs on Human Breast Carcinoma Xenografts in Nude Mice

Xenograft	HMM	MMC	ADM	CPA	5-FU	CDDP
T-61	0[a]	44.1	52.0	21.4[a]	ND	ND
MX-1	0[a]	7.9[a]	39.8[a]	0.8[a]	52.4	3.4[a]
MCF-7	88.5	23.4[a]	64.5	36.3[a]	63.5	ND
R-27	59.0	11.7[a]	70.5	36.3[a]	ND	ND
Br-10	40.6[a]	21.0[a]	90.0	30.9[a]	89.3	ND
Response rate	2/5	4/5	1/5	5/5	0/3	1/1

Data were shown as the lowest T/C ratio of the relative mean tumor weight during the experiments. Abbreviations: HMM, hexamethylmelamine 75 mg/kg for all tumors except T-61, 25 mg/kg for T-61 qd×24 p.o.; MMC, mitomycin C 6 mg/kg qd×1 i.p.; ADM, adriamycin 8 mg/kg qd×1 i.v.; CPA, cyclophosphamide 80 mg/kg q4d×3 i.p.; 5-FU, 5-fluorouracil 60 mg/kg q4d×3 i.p.; CDDP, cisplatin 9 mg/kg qd×1 i.p.

a) Positive antitumor effect (T/C equal to or less than 42%). ND: not done.

Table III. Effect of Drugs on Human Stomach and Colon Carcinoma Xenografts in Nude Mice

Xenograft		HMM	MMC	ADM	CPA	5-FU	CDDP
Stomach	SC-1-NU	32.8[a]	68	23[a]	ND	2[a]	40[a]
	St-4	10.7[a]	65.0	48.3	45.6	87.6	91.7
Colon	Co-3	31.5[a]	71.9	55.4	92.0	44.8	ND
	Co-4	31.3[a]	13.8[a]	52.1	81.0	13.4[a]	15.8[a]
	Co-6	41.5[a]	2.6[a]	69.4	ND	44.2	52.9
Response rate		5/5	2/5	1/5	0/3	2/5	1/3

Data were shown as the lowest T/C ratio of the relative mean tumor weight during the experiments. Abbreviations: HMM, hexamethylmelamine 75 mg/kg qd×24 p.o.; MMC, mitomycin C 6 mg/kg qd×1 i.p.; ADM, adriamycin 8 mg/kg qd×1 i.v.; CPA, cyclophosphamide 80 mg/kg q4d×3 i.p.; 5-FU, 5-fluorouracil 60 mg/kg q4d×3 i.p.; CDDP, cisplatin 9 mg/kg qd×1 i.p.
a) Positive antitumor effect (T/C equal to or less than 42%). ND: not done.

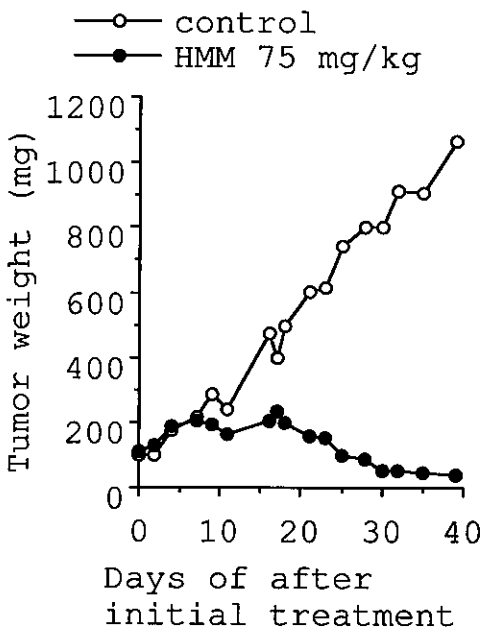

Fig. 6. Antitumor activity of hexamethylmelamine (HMM) on St-4 xenografts in nude mice. Treatment was initiated when the mean tumor weight reached 100 mg. HMM was administered per os for 4 weeks every day except Sundays.

Table IV. Toxicity of Hexamethylmelamine in Nude Mouse

Treatment			Maximum body wt. loss (%)[a]	Death rate[b]
Route	Dose (mg/kg)	Schedule		
p.o.	12.5	qd×24	7.1	0/6
	25	qd×24	12.4	0/6
	50	qd×24	2.6	0/14
	75	qd×24	15.9	0/19
	100	qd×6[c]	17.1	1/5
i.p.	200	q4d×2	31.0	5/5

a) Body weight loss = 1 − (mean body weight of treated mice/mean body weight of untreated mice).
b) Number of dead mice/number of treated mice.
c) The treatment had to be stopped because of weakness and body weight loss on day 6. One mouse died on day 22 after initial treatment.

DISCUSSION

Although HMM was first developed from cyanuric acid as an anticancer agent in the early 1950's,[7] this agent has been underestimated for a long time because its effect was evaluated as negative in the screening system using commonly available rodent tumors including L1210, P388 leukemias, Lewis lung carcinoma and B16 melanoma.[8] Recently, HMM was concluded to be clinically effective on ovarian, small cell-lung and breast carcinomas[8] in Western countries. One of the mechanisms of action of HMM is thought to be the covalent binding of its metabolites to tissue macromolecules such as DNA, RNA, or macromolecular proteins[9]; however, the mechanism involved has not been completely clarified.

Tables II and III indicate the correlation of antitumor activities of HMM and other antitumor agents on human tumor xenografts, showing the coefficient of correlation between the lowest T/C ratios of 10 human tumor xenografts treated with HMM or the other antitumor agents. The correlation coefficients were not statistically significant, suggesting that the antitumor spectrum of HMM is different from those of conventional agents, including mitomycin C (MMC), adriamycin (ADM), cyclophosphamide, 5-fluorouracil (5-FU) and CDDP, which are considered as first-line antitumor agents for breast, gastrointestinal tract, lung and ovarian carcinomas. This is consistent with the result that HMM does not show consistent collateral-sensitivity with other classical alkylating agents in preclinical tumor models.[10] This different antitumor spectrum of HMM might validate the clinical

application of this drug on ovarian cancer after failure of CDDP-based multiple-agent chemotherapy.[11] An early phase II study of HMM was started in July, 1993 in Japan on ovarian, lung and colon carcinomas. This drug might also be effective against tumors previously treated with conventional first-line drugs.

HMM was administered per os to nude mice, because it is insoluble in water. Its efficacy and side effects are influenced by the size of its crystals. This insolubility of HMM might be an advantage because active metabolites are released continuously after oral administration. Moreover, daily administration of this drug is thought to be favorable to maintain a relatively low concentration of the active metabolites in blood and thus to prevent the occurrence of side effects. Although oral agents are regarded as rather ineffective on solid tumors, as compared with intravenously administered drugs, HMM might be an exception. The side effects of HMM are nausea, vomiting and myelo-suppression, as well as peripheral nerve disorder, which is not usually observed with other anticancer agents.[12]

Of the five human breast carcinoma xenografts treated with HMM, MX-1 regressed completely at MTD, T-61 disappeared completely even at 1/3 MTD, and Br-10 was sensitive, whereas MCF-7 and R-27 were resistant to HMM at its MTD. HMM was also effective on all stomach and colon carcinoma xenografts employed in this study, whereas MMC, ADM, 5-FU and CDDP were effective against only one or two tumors. Two stomach cancer xenografts, SC-1-NU and St-4 were used for this study as sensitive and resistant strains to conventionally available antitumor agents, respectively, in line with our preclinical study.[4] St-4 was resistant to all the antitumor agents tested, including MMC and 5-FU, which are the first-line drugs for stomach cancer, and MMC was also ineffective on SC-1-NU. It is noteworthy that HMM proved very effective on these two strains: St-4 tumor regressed completely. Although stomach cancer is not included in the present clinical trial of HMM, this antitumor spectrum of HMM would warrant further preclinical and clinical studies of HMM for the treatment of stomach cancer.

The antitumor spectrum of HMM on human carcinoma xenografts was different from that of ADM, cyclophosphamide and CDDP, which are considered as representative antitumor agents.[1] Since HMM seems to be a promising agent for the treatment of patients with breast, stomach or colon carcinomas, further studies on its clinical usefulness and modes of action are warranted.

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