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Antitumor activity of a derivative of mitomycin, 7-*N*-[2-[[2-(γ-L-glutamylamino)ethyl]dithio]ethyl]mitomycin C (KW-2149), against murine and human tumors and a mitomycin C-resistant tumor in vitro and in vivo

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Summary. The antitumor activity of a mitomycin derivative, 7-*N*-[2[[2-(γ-glutamylamino)ethyl]dithio]ethyl]mitomycin C (KW-2149), was evaluated in murine and human tumor models, including a mitomycin C (MMC)-resistant tumor in vitro and in vivo. KW-2149 showed a profound effect against i.p. inoculated P388 leukemia on both a single and an intermittent administration schedule. Against s.c. implanted colon adenocarcinoma 38 (colon 38), KW-2149 was as effective as MMC in ILS% and in tumor growth inhibition on a single-administration schedule. Both compounds were similarly effective when an intermittent schedule was used. KW-2149 showed activity against human tumor xenografts and was effective in two of four non-small-cell lung carcinomas but was not effective against three gastric adenocarcinomas on the single-administration regimen. The activity of KW-2149 against gastric adenocarcinoma was inferior to that of MMC on a single-administration schedule. However, the antitumor activity of KW-2149 was higher on an intermittent schedule than on a single-administration regimen. The antitumor activity of KW-2149 against human tumor xenografts was similar to that of MMC on an intermittent schedule, and the former drug was effective against both gastric adenocarcinomas and both non-small-cell lung carcinomas. KW-2149 was more effective than MMC against a subline of P388 leukemia that is resistant to MMC in vitro as well as in vivo.

Introduction

Attempts have been made to synthesize new MMC derivatives by substituting a thiol side chain at the 7-*N* position so as to develop a compound with a broader antitumor spectrum that is less myelosuppresive than mitomycin C (MMC) [3, 6, 10, 14]. However, these efforts have thus for not been satisfactory. In an effort to develop new derivatives, we used a mode of action-oriented strategy for further development. The first step in the molecular mechanism of action of MMC has been considered to be the reduction of quinone moiety [12]. The observation that the quinone of mitomycin is easily reduced by a thiol and subsequently decomposed [11] envisages the derivatives containing thiol as being located at the side chain of the 7 position [7].

7-*N*-[2[[2-(γ-L-glutamylamino)ethyl]dithio]ethyl]mitomycin C (KW-2149) was synthesized, and its activity against P388 leukemia (i.p.–i.p.) and sarcoma 180 (s.c.–i.p.) is higher than that of MMC [8]. Thus far, no chemotherapeutic agent that shows satisfactory activity against carcinomas, especially non-small-cell lung carcinoma, gastric carcinoma, and colon carcinoma, has been developed. To this end, a further evaluation of KW-2149 was performed mainly in a solid murine tumor and in human tumor xenografts. To differentiate between KW-2149 and MMC, the former was evaluated in an MMC-resistant murine tumor.

Materials and methods

Drugs. KW-2149 was prepared as previously described [7]. An MMC formulation for clinical use was obtained from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan).

Animals. Male DBA/2Cr and C57BL/6J, C57BL/6J × DBA/Cr F_1 ($BD2F_1$) and BALB/c × DBA/2Cr F_1 ($CD2F_1$) mice were obtained from Charles River Japan Inc. (Tokyo, Japan). Female BALB/c-nu/nu athymic mice were obtained from Nihon Clea Inc. (Tokyo, Japan).

Murine tumors and evaluation of antitumor activity. P388 leukemia and colon adenocarcinoma 38 (colon 38) tumor cells were kindly provided by the National Cancer Institute of the United States. These tumors were maintained according to the protocol of the National Cancer Institute. P388 leukemia was maintained in male DBA/2Cr F_1 mice and colon 38 was maintained in male C57BL/6J mice. Sublines of P388 leukemia resistant to MMC (P388/MMC) [4], cyclophosphamide (P388/CPA), and cisplatin (P388/DDP), and the L1210 leukemia subline resistant to

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Table 1. Antitumor activity of KW-2149 against i.p. inoculated P388 leukemia

Drug	Schedule[a] (days)	Dose (mg/kg per day)	MS[b] (days)	ILS (%)	Tumor-free survivors	Body wt. change[c] (g)
Control			9.8±0.8	0	0/6	+1.2
KW-2149						
	1	36	9.8±6	0	0/6	−2.7
	1	30	10.2±4.9	4	1/6	−2.7
	1	25	22 ±3.6	124	2/6	−1.2
	1	20.8	22.2±8.2	127	1/6	−2.4
	1	17.4	25.5±2.1	160	2/6	−1.5
	1	14.5	20.3±1.9	140	0/6	−0.3
	1	12.1	22.3±5.5	127	0/6	−0.4
	1	10	20.3±1.2	107	0/6	−0.1
	1,5,9	11.9	25 ±5.2	155	0/6	−0.6
	1,5,9	10	24.3±1.4	147	0/6	−0.7
	1,5,9	8.3	23.8±3.2	153	0/6	+0.1
	1,5,9	6.9	23.7±3.9	142	0/6	−0.7
	1,5,9	5.8	22.3±1.4	127	0/6	−0.3
	1,5,9	4.8	20 ±2.5	104	0/6	+0.1
	1,5,9	4	17.7±0.5	80	0/6	+0.3
	1,5,9	3.3	20 ±1.8	104	0/6	+0.4

[a] 10^6 P388 leukemia cells were inoculated i.p. into $CD2F_1$ mice on day 0. KW-2149 was given i.p.

[b] Mean survival ± SD (duration of survival for mice that survived for >31 days was excluded from calculation)

[c] Body weight change from day 1 to day 5

cisplatin (L1210/DDP) were established in the Cancer Chemotherapy Center (Tokyo). These five tumors were maintained in male $CD2F_1$ mice.

For evaluation of antitumor activity, 10^6 P388 leukemia cells and 10^6 P388/MMC cells were injected i.p. into male $CD2F_1$ mice on day 0. KW-2149 and MMC were given i.p. on day 1; on days 1, 5, and 9 for P388 leukemia; and on day 1 for P388/MMC. Antitumor activity was determined by comparing the mean survival of the treated group (T) with that of the control group (C) and was expressed as the ILS%, i.e., (T/C-1) ×100%.

A colon 38 tumor fragment (2^3 mm^3) was inoculated s.c. into the flanks of $BD2F_1$ mice on day 0. KW-2149 and MMC were given i.v. either on day 2 or on days 2, 9, 16 and 23. Tumor diameters were measured with calipers once a week for 3 weeks, and the tumor volume (V) was calculated using the equation $V = {}^1/_2\, ab^2$, where a and b represent, respectively, the long and short diameters of the tumor mass in millimeters [2]. Tumor-free survivors, i.e., animals that had no microscopic P388 or P388/MMC tumor in their blood on day 31 and those with colon 38 that survived for >61 days, were excluded from calculations. Antitumor activity was determined by two factors: the percentage of mean tumor volume as compared with control values, with controls representing 100%, and the ILS%.

Human tumor xenograft model and experimental design. Human tumor xenografts used in these studies included three gastric adenocarcinomas (SC-6, NS-8, and SC-9), four non-small-cell lung carcinomas (Lu-99, QG-56, LC06, and LC11) [11], and two colon carcinomas (COL-1, COL-5). These tumors were established at the Central Institute for Experimental Animals (Kawasaki, Japan) and were maintained either in our Center or at the Central Institute.

For the experiments, xenograft fragments were implanted s.c. into the right subaxillary regions of nude mice. When the tumor had grown to 100–300 mm^3, the mice were randomized to several experimental groups consisting of six animals per group, and KW-2149 and MMC were given i.v. on the same day at doses of 17 and 6.7 mg/kg, respectively for the single-administration experiment. For the intermittent administration schedule (every 4 days × 3), KW-2149 and MMC were given i.v. at respective doses of 9.6 or 4.8 and 2.8 or 1.4 mg/kg daily. These high doses represented the maximum tolerated dose or one-half of the maximum tolerated dose of each drug on each schedule, which was determined in female BALB/C-nu/nu mice in a separate experiment. The volume of each tumor was calculated twice a week and expressed as relative tumor volume RV = Vn/Vo, where Vn represents the tumor volume on day n and Vo indicates the initial tumor volume at the beginning of the treatment (day 0).

The effectiveness of each drug was evaluated on day 14 using the equation:

$$T/C(\%) = \frac{\text{mean RV of the treated group}}{\text{mean RV of the control group}} \times 100.$$

Evaluation as "effective" was based on a T/C(%) of ≤50%, with statistical significance being determined according to the Mann-Whitney *U*-test ($P < 0.01$, one-sided).

Cell culture and drug treatment. Drug-sensitive and -resistant P388 and L1210 leukemia cells were harvested from the peritoneal cavity of tumor-bearing mice and were maintained in suspension culture in plastic dishes (Corning Glass Works, Corning, N.Y.) in RPMI 1640 medium supplemented with 5% fetal bovine serum, 20 μM 2-mercaptoethanol and 100 μg/ml kanamycin (growth medium) [13]. The cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. For the drug-treatment experiment, tumor cells (2×10^4) were cultured at 37°C for 6 h in growth medium in a humidified atmosphere containing 5% CO_2 and were treated with a graded concentration of KW-2149 and MMC. Cells were then reincubated for 72 h in the presence of the drugs and were counted with a model ZBI Coulter counter (Coulter Electronics, Hialeah, Flo.) [13]. The IC_{50} value was determined by plotting the logarithm of the drug concentration against the growth rate of treated cells to control cells (T/C).

Results

Antitumor activity of KW-2149 against murine tumors

Antitumor activity against i.p. inoculated P388 leukemia was determined using a single and an intermittent i.p. administration schedule. Maximal ILS values of 160% and 155%, respectively, were observed at 17.8 mg/kg for a single administration and 11.9 mg/kg daily for intermittent administration. One to two mice were cured at a dose of 17.4–30 mg/kg given in a single administration, but no mouse was cured on the intermittent schedule (Table 1). The results suggest that the activity of KW-2149 was affected by the administration schedule and that a single administration gave a better antitumor effect than did the intermittent schedule.

The antitumor activities of KW-2149 and MMC against colon 38 were evaluated by the ILS and the inhibition of tumor growth in tested mice. All animals treated with KW-2149 survived for >61 days at doses of 12.5 and 6.25 mg/kg daily given by single and intermittent administration, respectively. In the MMC-treated group, all six mice survived for >61 days at single doses of 4 and 6 mg/kg daily and at 2 mg/kg daily given by intermittent administration. At the prominently effective doses of these drugs, two to three of the six mice became tumor-free on these treatment schedules. KW-2149 and MMC also significantly suppressed tumor growth. KW-2149 toxicity was observed at 25 mg/kg for a single dose and at 18.8 mg/kg daily for intermittent administration (Table 2).

Table 2. Antitumor activity of KW-2149 and MMC against colon adenocarcinoma 38

Drug	Schedule[a] (days)	Dose (mg/kg per day)	MS[b] (days)	ILS (%)	Long-term survivors[c]	tumor volume (mm^3)	Growth[d] T/C (%)
Control			40.1± 7	0	0/6	1,511	100
KW-2149	2	25	8	–80	4/6	0	–
		18.8	34.5±21.5	–14	4/6	0	–
		12.5	–		6/6	211	14
		6.3	56 ± 1.6	40	3/6	823	55
MMC	2	6	–		6/6	170	11
		4	–		6/6	186	12
		2	58 ± 2	45	4/6	711	47
KW-2149	2,9,16,23	18.8	14 ± 7	–65	2/6	0	–
		12.5	41	2	5/6	0	–
		6.3	–		6/6	586	39
		3.1	56.2± 4.4	40	0/6	893	59
MMC	2,9,16,23	6	31.7±15.1	–21	0/6	0	–
		4	38	– 7	5/6	56	4
		2	–		6/6	481	32
		1	53.7± 4.2	34	3/6	509	34

[a] Colon adenocarcinoma 38 tumor (2×2×2 mm) was inoculated s. c. into the flank of $BD2F_1$ mice on day 0
[b] Mean survival ± SD (duration of survival for mice that survived for >61 days was excluded from calculation)
[c] Mice surviving for >61 days/mice treated
[d] Tumor volume was determined on day 21

Table 3. Antitumor activity of KW-2149 and MMC against human tumors xenografted into nude mice

Tumor xenograft		T/C[a] (%)	
		KW-2149	MMC
Gastric adenocarcinoma:			
	SC-6	61 (–2.2)[b]	12[c] (–2)[b]
	NS-8	62 (–3.3)	64 (–4.1)
	SC-9	52 (–3.2)	18[c] (–3.7)
Lung carcinoma:			
	Lu-99	26[c] (–4)	25[c] (–6)
	QG-56	57 (–2.4)	52 (–3.5)
	LC-6	38[c] (–2.9)	58 (–4.5)
	LC-11	56 (–2.7)	49 (–4.4)
Colon carcinoma:			
	COL-1	59 (–4.7)	72 (–3.1)
	COL-15	72 (–5.4)	54 (–6.7)

[a] Fragments of human tumor were implanted s. c. in the flank of athymic BALB/c-nu/nu mice. When the tumor volume reached 100–300 mm^3, KW-2149 (17 mg/kg) and MMC (6.7 mg/kg) were injected i. v. and tumor volume was determined on day 14
[b] Numbers in parentheses show the maximal change in body weight (in grams) over 14 days
[c] T/C(%) <50 and P <0.01 (one-sided) according to the Mann-Whitney U-test, as compared with control values

Table 4. Antitumor activity of KW-2149 and MMC given by intermittent schedule against human tumors xenografted into nude mice

Tumor xenograft		KW-2149			MMC		
		Dose[a] (mg/kg per day)	T/C[b] (%)	Body wt. change[c] (g) (day)[d]	Dose[a] (mg/kg per day)	T/C[b] (%)	Body wt. change[c] (g) (day)[d]
Gastric adenocarcinoma:							
	NS-8	9.6	43[e]	–4 (10)	2.8	51	–2.3 (10)
		4.8	57	–1.8 (10)	1.4	65	–1.3 (10)
	SC-9	9.6	32[3]	–4 (14)	2.8	30[e]	–4 (11)
		4.8	51	–2.3 (18)		NT	
Lung carcinoma:							
	LC-6	9.6	28[e]	–3.4 (14)	2.8	3[e]	–2.5 (10)
		4.8	57	–1.3 (14)		NT	
	LC-11	9.6	36[e]	–1.1 (4)	2.8	46[e]	–1 (4)
		4.8	55	–0.8 (4)	1.4	65	–0.6 (4)

[a] When the tumor volume reached 100–300 mm^3, KW-2149 and MMC were given i. v. every 4 days for a total of three injections
[b] Tumor volume was determined on day 14
[c] Body weight change after injection of drugs
[d] Day of maximal decrease in body weight
[e] T/C(%) <50 and P <0.01 (one-sided) according to the Mann-Whitney U-test
NT, Not tested

Antitumor activity of KW-2149 against human tumor xenografts

The antitumor activity of KW-2149 and MMC against s. c. implanted human tumors is summarized in Tables 3 and 4. On the single-injection schedule, the activity of KW-2149 was inferior to that of MMC against three gastric adenocarcinomas. According to the evaluation criteria described in Materials and methods, KW-2149 was not effective against xenografts SC-6, NS-8, and SC-9, but MMC was effective against SC-6 and SC-9. KW-2149 was effective against two of four xenografts non-small-cell carcinoma (Lu-99 and Lc-6), whereas MMC was effective against only Lu-99. Neither drug showed antitumor activity against colon cancers COL-1 and COL-5 (Table 3).

Table 5. In vitro cytotoxicity of KW-2149 and MMC to sensitive and resistant leukemia cells

Tumor cells	IC_{50}[a] (nM)	
	KW-2149	MMC
P388	9 ± 0.3	48 ± 0.2
P388/MMC	37 ± 1 (4.1)[b]	583 ± 65 (12.1)[b]

[a] IC_{50} after 72 h incubation with drug; values were expressed as the mean ± SD of triplicate determinations

[b] Numbers in parentheses show the ratio of the IC_{50} value for resistant cells to that for sensitive cells

Table 6. Antitumor activity of KW-2149 against MMC-resistant P388 leukemia

Drug	Dose[a] (mg/kg)	MS[b] (days)	ILS (%)	Tumor-free survivors[c]	Body wt. change[d] (g)
Control		14.4 ± 3.9	0		−0.3
KW-2149	25	9.8 ± 1.9	−32	2/6	−2.6
	12.5	17.3 ± 3	20	2/6	−0.8
	6.25	18.4 ± 2.4	27	1/6	−0.9
	3.12	13.8 ± 5.2	−4	0/6	−0.3
	1.56	16 ± 3	11	0/6	0.0
MMC	8	19.7 ± 6.1	36	0/6	−1.2
	6	18 ± 2.8	25	0/6	−0.8
	4	15.8 ± 4.1	10	0/6	−2.0
	2	12.8 ± 3.1	−11	0/6	−0.7

[a] 10^6 P388/MMC leukemia cells were inoculated i.p. into female $CD2F_1$ mice (6 mice/group) on day 0. KW-2149 and MMC were given on day 1

[b] Mean survival ± SD for the calculation of MS; long-term survivors (>31 days) were omitted from the calculation

[c] Long-term survivors (>31 days) with non-palpable tumors at the inoculation site and in the blood

[d] Body weight change from day 1 to day 5

Because the antitumor activity of KW-2149 had previously been shown to be higher on the intermittent schedule in the i.v.–i.v. system of P388 leukemia [9], both drugs were given in two intermittent doses on days 0, 4, and 8. Against NS-8, KW-2149 and MMC gave T/C values of 43% and 51%, respectively, and the antitumor activity of KW-2149 might be higher on an intermittent schedule than with a single administration. Against SC-9, KW-2149 and MMC were effective at T/C values of 32% and 30%, respectively. Againt LC-6, KW-2149 showed activity at a T/C value of 28% but was less active than MMC. Against LC11, KW-2149 and MMC showed T/C values of 36% and 46%, respectively. In summary, KW-2149 was effective against all four xenografts, whereas MMC was effective against only three (Table 4).

Activity of KW-2149 against the MMC-resistant leukemia subline

The growth-inhibitory activity of KW-2149 and MMC against the MMC-resistant P388 leukemia subline was evaluated in culture. P388/MMC showed 12.5-fold resistance to MMC when the ratio of IC_{50} values for resistant vs sensitive cells were compared (Table 5). However, as compared with P388 leukemia cells, P388/MMC cells showed only 4.1-fold resistance to KW-2149. These results indicate that KW-2149 is not completely cross-resistant to MMC and might be effective against some MMC-resistant tumors. P388/CDDP, P388/CPA, and L1210/CDDP cells showed resistance to KW-2149 comparable with that to MMC (data not shown).

The antitumor activities of KW-2149 and MMC against i.p. inoculated P388/MMC cells were examined on a single-administration schedule. At a maximal dose (8 mg/kg) MMC gave 36% ILS, but long-term survival was not observed. However, at 12.5 and 6.25 mg/kg, KW-2149 showed ILS values of 20% and 27%, respectively. Of the six mice, three were rendered tumor-free: two at a dose of 12.5 mg/kg and one at 6.25 mg/kg (Table 6). The superior activity of KW-2149 over MMC against P388/MMC cells in vivo could be explained by its superior in vitro cytotoxicity.

Discussion

KW-2149, a derivative of MMC with disulfide at the 7-*N* position, was selected for its superior antitumor activity over MMC against solid sarcoma 180 and P388 ascites leukemia [8]. We evaluated the antitumor activity of KW-2149 in solid murine tumors, human tumor xenografts, and MMC-resistant P388 leukemia. The compound produced significantly increased survival in mice implanted with P388 leukemia using the i.p.–i.p. system. Against solid colon 38 tumor, KW-2149 was as active as MMC in achieving the maximal ILS, in the number of mice cured, and in inhibiting tumor growth. Its antitumor activity against human tumor xenografts was evaluated using single and intermittent administration schedules. KW-2149 proved to be effective against both gastric tumor xenografts and both non-small-cell lung carcinomas. These results might suggest that the antitumor spectrum of KW-2149 is as wide as that of MMC.

The administration schedule that resulted in optimal antitumor activity varied among the systems tested. Against i.p. implanted P388 leukemia and s.c. implanted colon 38, the single-administration schedule resulted in significant activity that was superior to or comparable with the intermittent schedule. Against s.c. implanted human tumor xenografts, intermittent administration was more effective for growth inhibition.

One of the interesting characteristics of KW-2149's activity was its effectiveness against P388/MMC leukemia cells. The IC_{50} value for MMC in P388/MMC was 12.5-fold that in P388 leukemia cells, whereas P388/MMC were only 4.1-fold more resistant to KW-2149 than were P388 leukemia cells. This in vitro result could explain the effectiveness of KW-2149 against i.p. inoculated P388/MMC when the drug was given by the same route. It also suggests that the mechanisms of activity of KW-2149 are somewhat different from those of MMC. One of the derivatives of MMC with an amidine substituted at 7-*N* position (BMY25282) has also been reported to be capable of overcoming MMC resistance in human colon carcinoma cells resistant to MMC [16], and it produced significant differ-

ences in the formation of interstrand DNA cross-links between MMC-sensitive and -resistant cells incubated with BMY25282. These authors suggested that the resistant cells showed a low level of MMC activation as compared with sensitive cells. KW-2149 may be useful for studies of the resistant mechanism of MMC.

The growth-inhibitory activity of KW-2149 was 5- to 7-fold that of MMC against P388 leukemia cells in culture. On the other hand, the optimal dose of KW-2149 was about 3-fold that of MMC in tumor-bearing mice. The characteristics of low toxicity in vivo and high activity in vitro suggest that KW-2149 can be applied not only for systemic treatment but also for local application in clinical therapy.

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