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Characteristics of the antitumor activity of M-16 and M-18, major metabolites of a new mitomycin C derivative KW-2149, in mice

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The cell growth inhibitory activity, antitumor activity and toxicity of M-16 and M-18, the major metabolites of a new mitomycin C (MMC) derivative KW-2149, in mouse and human were compared with those of KW-2149 or MMC *in vitro* and *in vivo*. The growth inhibitory activity of M-18, a symmetrical disulfide dimer, against human uterine cervix carcinoma HeLa S_3 cells was almost equivalent to that of KW-2149 and their IC_{50} values were about 10-fold smaller than that of MMC. The activity of M-16, a methyl sulfide form, was almost equivalent to that of MMC. The cell-killing activity of MMC and M-16 was augmented in the hypoxic condition, whereas that of KW-2149 and M-18 was reduced. M-16 also exhibited almost equivalent activities to MMC *in vivo* in terms of many biological profiles, i.e. antitumor activity against murine P388 leukemia, ascitic or solid B16 melanoma or human lung carcinoma xenograft L-27, and bone marrow toxicity in mice. These *in vitro* and *in vivo* results indicate that the antitumor activity and toxicity of KW-2149 might not be mediated by M-16 in mice. On the other hand, M-18 exhibited almost equivalent activities to KW-2149 in these regards, suggesting the involvement of M-18 in the biological activities of KW-2149. However, the small values of the area under the curve of M-18 in mice make this unlikely. Thus the biological activities of KW-2149 in mice are not explained by the M-16 or M-18 concentration in plasma and are postulated to be manifested by KW-2149 itself.

Key words: KW-2149, mitomycin C, metabolite.

Introduction

7-*N*-{[2-{[2-(γ-L-glutamylamino) ethyl]dithio}ethyl]}-mitomycin C (KW-2149) is a newly synthesized water-soluble analog of mitomycin C (MMC) and was selected for clinical study based on its broad antitumor activities equal or superior to those of MMC in many experimental systems.[1–5] KW-2149 was also effective in increasing the life span of mice bearing P388 leukemia resistant to MMC (hereafter designated as P388/MMC).[4,5] KW-2149 was comparable to MMC in decreasing the number of white blood cells (WBC) in the peripheral blood of mice, but the thrombocytopenia induced by KW-2149 was mild and recovered rapidly.[5,6] The *in vitro* anticellular spectrum of KW-2149 against 24 human tumor cell lines was similar to that of MMC.[5] However, KW-2149 inhibited the growth of various cell lines at 10 to 100 times lower concentrations than MMC.[5] This characteristic of KW-2149 is suggested to be explained by its more efficient uptake into the cells than MMC, as demonstrated in murine P388 leukemia cells.[7] MMC is activated enzymatically by NADPH:cytochrome P450 reductase, DT-diaphorase, xanthine oxidase or xanthine dehydrogenase,[8–11] and such bioreductive activation might be essential for its antitumor activity. Interestingly, KW-2149 was found to be activated non-enzymatically by thiol molecules like glutathione or cysteine, and this mechanism is suggested to be associated with its effectiveness against MMC-resistant tumors.[12] Furthermore, KW-2149 bound covalently with both DNA and protein, and this may also lead to the potent and characteristic antitumor activity of KW- 2149 as compared with MMC.[13]

Recently the disposition and metabolism of KW-2149 were examined in both mouse and human.[14,15] The blood level of KW-2149 decreased rapidly in mice following its intravenous administration and two major metabolites, M-16 and M-18 (Figure 1), became detectable.[14,15] The *in vitro* cell killing activity of the metabolite M-18 was reported to be significant and comparable with that of KW-2149.[16] These results induced us to compare the antitumor activity and toxicity of these metabolites with those of KW-2149 or MMC in mice.

Materials and methods

Chemicals

KW-2149, M-16, M-18 and MMC were produced in our laboratories. For *in vitro* experiments, all com-

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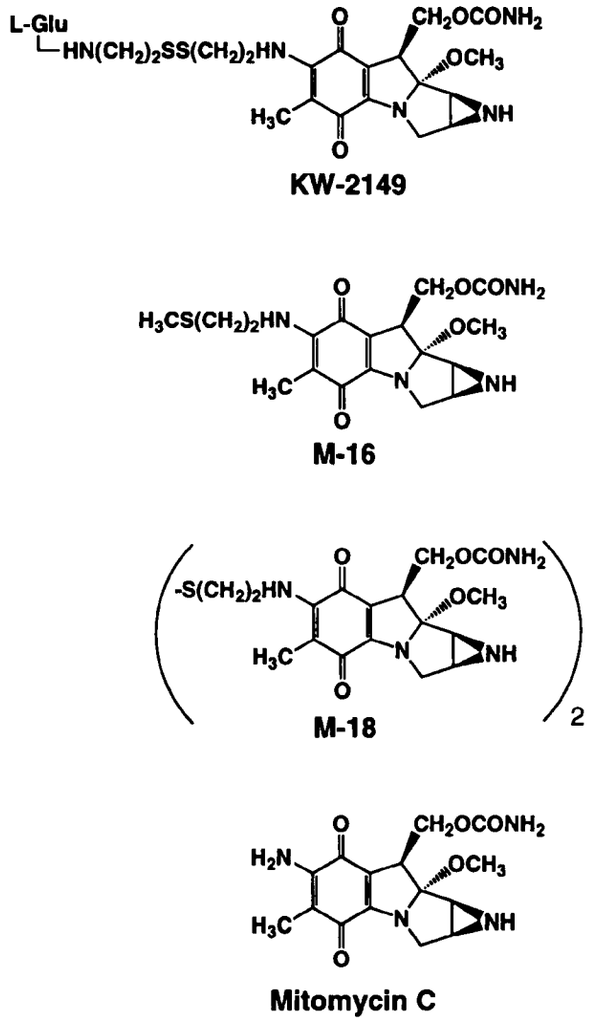

Figure 1. Structure of major metabolites of KW-2149 in mice.

pounds were dissolved in dimethyl sulfoxide (DMSO) and diluted with the culture medium to a final concentration of DMSO not exceeding 0.5% (v/v). For *in vivo* experiments, KW-2149 and MMC were dissolved in 0.9% (w/v) NaCl solution, and M-16 and M-18 in 0.9% (w/v) NaCl solution containing Tween 80. The ratios of Tween 80 to M-16 and M-18 were 10 and 20 μl/mg, respectively.

Cell growth inhibitory activity

HeLa S_3 cells were obtained from American Type Culture Collection via Dainihon Pharmaceutical (Osaka, Japan). The cells (3×10^3/well) were precultured for 24 h in 96-well microplates (Nunc, Roskilde, Denmark) containing 0.1 ml of Eagle's minimal essential medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY) (hereafter culture medium) at 37°C in a humidified atmosphere containing 5% CO_2 in air. The cells were then treated with each compound for 1 h, washed and further incubated for 71 h in the fresh culture medium. The anticellular activity of compounds was evaluated by the inhibition of the uptake of neutral red dye into the cells. Briefly, after the above incubation, the culture medium was discarded and 0.1 ml of 0.02% (w/v) neutral red-containing medium was added to each well. After incubation for 1 h, the solution was discarded and each well was washed with 0.1 ml of 0.9% (w/v) NaCl solution. The neutral red dye was extracted by 0.1 ml of 30% (v/v) ethanol solution with 0.001 N HCl and the absorbance at 550 nm was measured by Microplate Reader (Corona Electric, Ibaragi, Japan). The anticellular activity was expressed by IC_{50} values (concentration required for 50% inhibition of dye uptake).

Cell killing activity

The cell killing activity of drugs was determined as follows.[17] HeLa S_3 cells (10^5) were precultured for 24 h in plastic flasks (Nunc) containing 10 ml of culture medium at 37°C in a humidified atmosphere containing 5% CO_2 in air in the aerobic condition. To produce hypoxic conditions, flasks were sealed with rubber stopper and exposed to continuously flowing 95% nitrogen/5% CO_2 (O_2 0.2 p.p.m.) through the needle for 4–5 h at 37°C. The cells in these aerobic or hypoxic conditions were treated for 1 h with each compound by injection with a needle through the rubber stoppers. The monolayer cells were then washed, subjected to the free cells by treatment with 0.02% (w/v) EDTA and further incubated for 7 days in fresh culture medium. The number of colonies was counted by staining them with 0.5% (w/v) crystal violet containing 10% (v/v) formaldehyde and 20% (v/v) ethanol.

Animals and tumors

Sarcoma 180 cells were passaged and used for the experiment in adult male ddY mice weighing 19–25 g. Murine lymphocytic leukemia P388 and P388/MMC cells were passaged in adult male DBA/2 mice and used for the experiment in adult male BALB/c × DBA/2 F_1 ($CD2F_1$) mice weighing 20–25 g. B16

melanoma cells were passaged and used in adult male C57BL/6 mice weighing 20–25 g. These animals were obtained from Japan SLC (Shizuoka, Japan). Human lung adenocarcinoma xenograft L-27 and breast adenocarcinoma xenograft MX-1 were passaged and used in adult male BALB/c-*nu/nu* mice (hereafter nude mice) weighing 20–25 g obtained from Clea Japan (Tokyo, Japan). Sarcoma 180 was kindly supplied by the National Cancer Center (Tokyo, Japan); P388 leukemia and B16 melanoma by Dr T Tsuruo (Japanese Foundation for Cancer Research, Tokyo, Japan); P388/MMC leukemia[18] by Dr M Inaba (Japanese Foundation for Cancer Research); and human xenografts L-27 and MX-1 by Dr Y Ohnishi (Central Institute for Experimental Animals, Kanagawa, Japan).

Antitumor activity

Sarcoma 180 cells were inoculated subcutaneously at the axillary regions of ddY mice, and B16, L-27 and MX-1 in the flanks of C57BL/6 mice or nude mice. For the evaluation of antitumor activity against subcutaneously inoculated tumors, the tumor volume was calculated by the following formula according to the method of the National Cancer Institute[19] after measuring the lengths and widths of the tumors:

$$\text{Tumor volume (mm}^3\text{)} = \frac{\text{length (mm)} \times [\text{width (mm)}]^2}{2}.$$

The criteria for effectiveness against murine solid tumors are a T/C(%) value of 50 and less, and statistical significance determined by Mann–Whitney's *U*-test ($p < 0.05$).

Drug efficacy against L-27 and MX-1 inoculated into nude mice was expressed as the percentage of the mean V/V_0 value against that of the control group, where V is the tumor volume on the day of evaluation and V_0 is the tumor volume on the day of initial administration of each compound. The criteria for effectiveness were a T/C(%) value of 50 and less, and statistical significance determined by Mann–Whitney's *U*-test ($p < 0.01$, one-sided).

The antitumor activity against intraperitoneally inoculated tumors was evaluated by calculating the percentage of increase in life span (ILS(%)). The criteria for effectiveness were an ILS(%) value of 25 and more, and statistical significance determined by Mann–Whitney's *U*-test ($p < 0.05$).

Toxicity testing

The survival of young male ddY mice (4–6 weeks old, 19–21 g) administered with each compound was observed for 30 days, and the LD_{50} and LD_{10} values were calculated by probit analysis. The hematological toxicity was examined in mature female ddY mice (20–26 weeks old, 35–40 g) by measuring the number of WBC and platelets in peripheral blood which was collected from the retro-orbital sinus.

Results

Cell growth inhibitory and cell killing activity

The cell growth inhibitory activity of M-16 and M-18 was compared with that of KW-2149 or MMC against human uterine cervix carcinoma HeLa S_3 cells at 1 h exposure by the neutral red dye uptake method (Table 1). The activity of M-18 was almost equivalent to that of KW-2149 and the IC_{50} values of both compounds were about 10-fold smaller than that of MMC. The activity of M-16 was almost equivalent to that of MMC.

The cell killing activity of M-16 and M-18 was compared with that of KW-2149 or MMC in aerobic or hypoxic conditions (Table 1 and Figure 2). The order of cell killing activity of four compounds was

Table 1. Cell growth inhibitory activity and cell killing activity of KW-2149 and its metabolites, M-16 and M-18

Compounds	IC_{50} (μM)		
	growth inhibitory activity[a]	cell killing activity[b]	
	aerobic	aerobic	hypoxic
KW-2149	0.018	0.11	0.66
M-16	1.7	2.7	0.31
M-18	0.011	0.12	0.68
MMC	0.83	1.7	1.3

[a] HeLa S_3 cells (3×10^3/well) were cultured on day 0, treated with various concentrations of each compound for 1 h on day 1 and further incubated for 71 h in the fresh medium. IC_{50} values were measured by the inhibition of uptake of neutral red dye into the cells.

[b] HeLa S_3 cells (10^5/flask) were cultured on day 0 in the aerobic condition. On day 1, some flasks were moved to the hypoxic condition for 4 h and the cells on each condition were treated with various concentrations of each compound for 1 h. Then the monolayer cells were subjected to free cells, transferred to the dishes and further incubated. On day 8, the number of colonies was counted and IC_{50} values were calculated.

similar to that of their cell growth inhibitory activity, i.e. M-18 was as active as KW-2149, and M-16 as MMC. Interestingly, the cell killing activity of MMC and M-16 was augmented under hypoxic conditions, whereas that of KW-2149 and M-18 was reduced, suggesting that the latter two compounds are activated by somewhat different mechanisms compared with the former two compounds.

Antitumor activity against murine and human tumors

The *in vivo* antitumor activity of M-16 and M-18 was compared with that of MMC or KW-2149 in mice (Tables 2 and 3, and Figure 3), and summarized in Table 4. M-18 was effective against intraperitoneally inoculated P388 leukemia, P388/MMC leukemia and B16 melanoma, and also subcutaneously inoculated sarcoma 180, B16 melanoma, human lung adenocarcinoma L-27 and breast adenocarcinoma MX-1. These characteristics of M-18 against P388, B16 melanoma and L-27 were relatively similar to those of KW-2149, and were superior to those of MMC. The characteristics of antitumor activity of M-16 were similar to those of MMC, although it was more active than MMC against subcutaneously inoculated sarcoma 180 and B16 melanoma. KW-2149, M-16 and M-18 were effective in increasing the life span of MMC-resistant P388 leukemia-bearing mice (Table 4).

The growth pattern of human lung adenocarcinoma L-27 and breast adenocarcinoma MX-1 inoculated into nude mice is shown in Figure 3. The growth inhibitory activity of KW-2149 or M-18 was more significant than that of MMC or M-16 against L-27, whereas their activity was less than that of MMC or M-16 against MX-1, suggesting again that the characteristics of antitumor activity of M-18 are relatively similar to those of KW-2149 and those of M-16 to MMC.

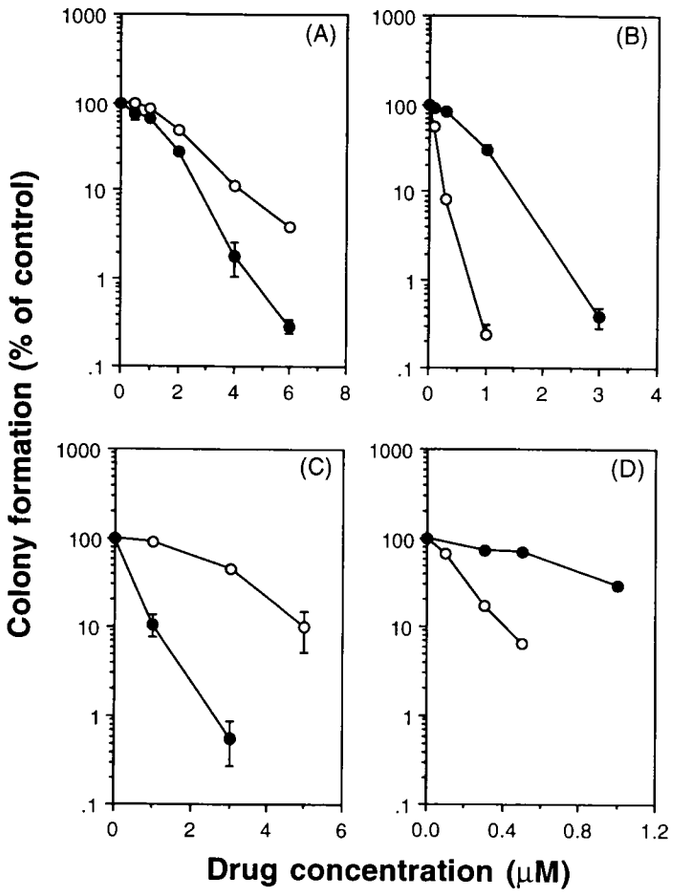

Figure 2. Cell killing activity of MMC (A), KW-2149 (B), M-16 (C) and M-18 (D) against human uterine cervix carcinoma HeLa S_3 cells in hypoxic (●) or aerobic (○) condition at 1 h exposure. Mean ± SD.

Toxicity

The lethal toxicity of M-16 or M-18 is compared with those of KW-2149 or MMC in young male ddY mice (Table 4). The LD_{50} and LD_{10} values of M-16 were nearly equivalent to those of KW-2149, while those of M-18 were nearly equivalent to those of MMC. The number of WBC and platelets in peripheral blood was measured periodically after intravenous administration of each compound in 20–26 week old mature female ddY mice, which had been revealed to be subject to the bone marrow toxicity of MMC,[6] on day 0, 7, 14 and 21 (Figure 4), and its percentages compared with those of untreated mice at the nadir are shown in Table 4. The nadir of WBC number was observed 3–4 days after each administration (Figure 4) and the degree of leukopenia induced by all four compounds was almost equal. On the other hand, some differences were observed in the degree of thrombocytopenia induced by the four compounds. MMC caused the severe thrombocytopenia and that induced by M-16 was also considerable, while that induced by KW-2149 and M-18 was not significant.

Discussion

In this study, the antitumor activity and toxicity of M-16 and M-18, the major metabolites of KW-2149 in both mouse and human, were evaluated *in vitro* and *in vivo*. In terms of the cell killing activity, Dirix *et al.* had already reported that M-18 was significant and comparable with KW-2149.[16] This result was

Table 2. Antitumor activity of KW-2149 and its metabolites against murine ascitic tumors

Tumors	Compounds	Dose (mg/kg)	Mean survival (days ± SD)	ILS (%)	>60 days survivors
P388	—	0	9.4 ± 0.5	0	0/5
	KW-2149	17	27.2 ± 14[a]	189	0/5
	M- 16	9.0	20.4 ± 3.0[a]	117	0/5
	M- 18	14	25.4 ± 3.2[a]	170	0/5
	MMC	8.0	19.6 ± 1.9[a]	109	0/5
B16	—	0	16.4 ± 2.2	0	0/5
	KW-2149	17	> 55.2 ± 11[a]	> 237	4/5
	M-16	4.5	28.0 ± 1.9[a]	71	0/5
	M-18	14	38.6 ± 13[a]	135	0/5
	MMC	4.0	33.0 ± 5.3[a]	101	0/5

P388 leukemia (1×10^6/mouse) and B16 melanoma (0.5 ml/mouse of 10% homogenate) cells were inoculated intraperitoneally on day 0. Drugs were injected intraperitoneally on day 1.
[a] $p < 0.05$ by Mann–Whitney's *U*-test as compared with the control group.

Table 3. Antitumor activity of KW-2149 and its metabolites against murine solid tumors

Tumors	Compounds	Dose (mg/kg)	Tumor volume (mm^3, mean ± SD)	T/C (%)	On day
Sarcoma 180	—	0	1681 ± 421	100	7
	KW-2149	17	132 ± 80[a]	7.9	7
	M-16	9.0	83 ± 21[a]	4.9	7
	M-18	14	587 ± 264[a]	35	7
	MMC	4.2	986 ± 338	59	7
B16	—	0	3201 ± 1032	100	19
	KW-2149	17	63 ± 10[a]	2.0	19
	M-16	12	830 ± 392[a]	26	19
	M-18	14	308 ± 221[a]	9.6	19
	MMC	4.2	1807 ± 727	57	19

Sarcoma 180 (5×10^6/mouse) and B16 melanoma (0.1 ml/mouse of 20% homogenate) cells were inoculated subcutaneously on day 0. Drugs were injected intravenously on day 1.
[a] $p < 0.05$ by Mann–Whitney's *U*-test as compared with the control group.

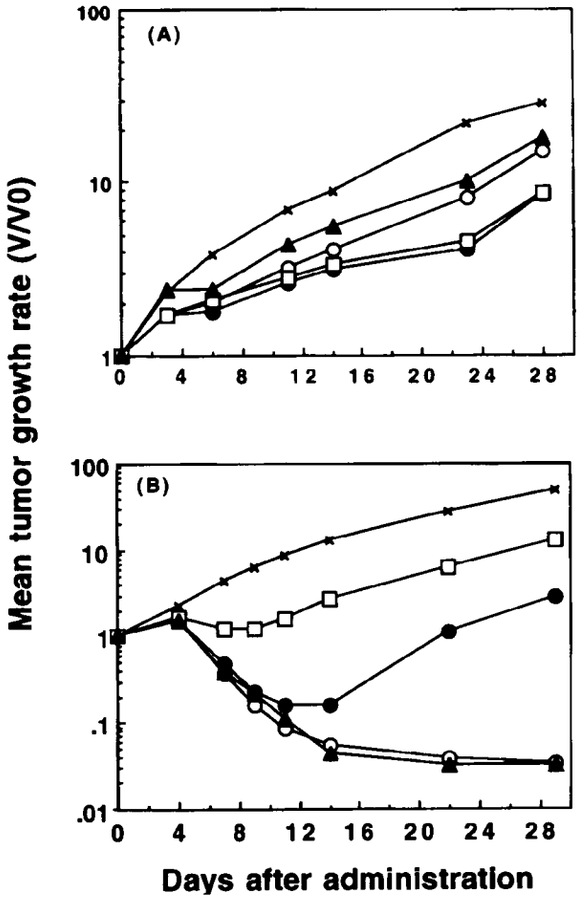

Figure 3. Growth pattern of human lung adenocarcinoma L-27 (A) or human breast adenocarcinoma MX-1 (B) inoculated into nude mice. KW-2149 (●, 17 mg/kg), M-16 (○, 12 mg/kg), M-18 (□, 6.7 mg/kg) or MMC (▲, 4.2 mg/kg) was injected intravenously on day 0. Untreated (×).

reproduced against human uterine cervix carcinoma HeLa S_3 cells in terms of the cell growth inhibitory activity, i.e. the IC_{50} values of KW-2149 and M-18 were about 10- fold smaller than those of MMC or M-16 in the aerobic condition (Table 1). The comparison of the cell killing activity of these four compounds in the aerobic condition also gave similar results (Table 1 and Figure 2). Furthermore, the cell killing activity of MMC and M-16 was augmented under hypoxic conditions, whereas that of KW-2149 and M-18 was reduced (Figure 2), suggesting that the latter two compounds were activated by somewhat different mechanisms from the former two compounds. KW-2149 and M-18 possess a disulfide bond within their molecules and this may contribute to their intrinsic biological activity.

One of major activation mechanisms of MMC is mediated by DT-diaphorase, which acts preferably in the hypoxic condition compared with the aerobic condition.[8] On the other hand, KW-2149 was reported to be activated non-enzymatically by thiol molecules like glutathione or cysteine, and DT-diaphorase was not involved in the mechanisms of its antitumor activity.[12] This may be associated with its reduced cell killing activity in the hypoxic condition (Figure 2). Once KW-2149 is activated by the cellular thiol molecules and chemically reduced to a sulfur ion, the sulfur ion may activate the quinone ring of KW-2149 to the semiquinone.[12] The oxygen provided under aerobic conditions may facilitate this activation process of KW-2149. In the phase I study of KW-2149, the pulmonary toxicity was the dose-limiting factor,[15] which might be associated with such an activation mechanism of KW-2149.

The LD_{10} value of M-16 was nearly equivalent to that of KW-2149 and that of M-18 to MMC (Table 4). This toxicological profile of M-16 and M-18 may be important to assess the biological significance of these two compounds in KW-2149-treated mice. After the administration of KW-2149 to both mouse and human, its plasma concentration decreased rapidly and two major metabolites, M-16 and M-18, became detectable.[14,15] The values of area under the curve of KW-2149, M-16 and M-18 were 111, 117 and 6.6 μg min/ml, respectively, in mice. This result suggests that most of the toxicological profiles of KW-2149 may be explained by the influence of its metabolite M-16. However, the profiles of biological activity of KW-2149 show similarity to M-18 rather than to M-16 in terms of antitumor activity and toxicity (Table 4). Therefore, we suppose that the characteristics of antitumor activity and toxicity of KW-2149 may be manifested mainly by KW-2149 itself, although M-16 and M-18 may have some role in them. Besides M-16 and M-18, the albumin conjugates of KW-2149, presumably formed by disulfide bonds, were also detected in the plasma of mice.[14] The biological significance of these conjugates should be examined in the future.

Conclusion

The *in vitro* cell growth inhibitory activity, *in vivo* antitumor activity and toxicity of M-16 and M-18, the major metabolites of KW-2149 in mouse and human, suggest that KW-2149 itself manifests major biological activities.

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Table 4. Summary of *in vivo* biological activity of KW-2149 and its major metabolites

Items	Compounds			
	KW-2149	M-16	M-18	MMC
Toxicity in ddY mice (i.v. administration)				
LD_{50} (mg/kg)	20	34	7.1	5.5
LD_{10} (mg/kg)	17	18	6.7	4.2
WBC number (%) at nadir	50	43	60	45
platelet number (%) at nadir	88	47	75	58
Antitumor activity				
ILS(%) against ascitic tumors (i.p. administration)				
P388 leukemia	189[a]	117[a]	170[a]	109[a]
P388/MMC leukemia	>126[a]	83[a]	63[a]	−7
B16 melanoma	>237[a]	71[a]	135[a]	101[a]
T/C(%) against solid tumors (i.v. administration)				
sarcoma 180	7.9[a]	4.9[a]	35[a]	59
B16 melanoma	2.0[a]	26[a]	9.6[a]	57
xenograft L-27	29[b]	45[b]	29[b]	61
xenograft MX-1	1.2[b]	0.1[b]	20[b]	0.1[b]

[a] $p < 0.05$ or [b] $p < 0.01$ (one-sided) by Mann–Whitney's *U*-test as compared with the control group.

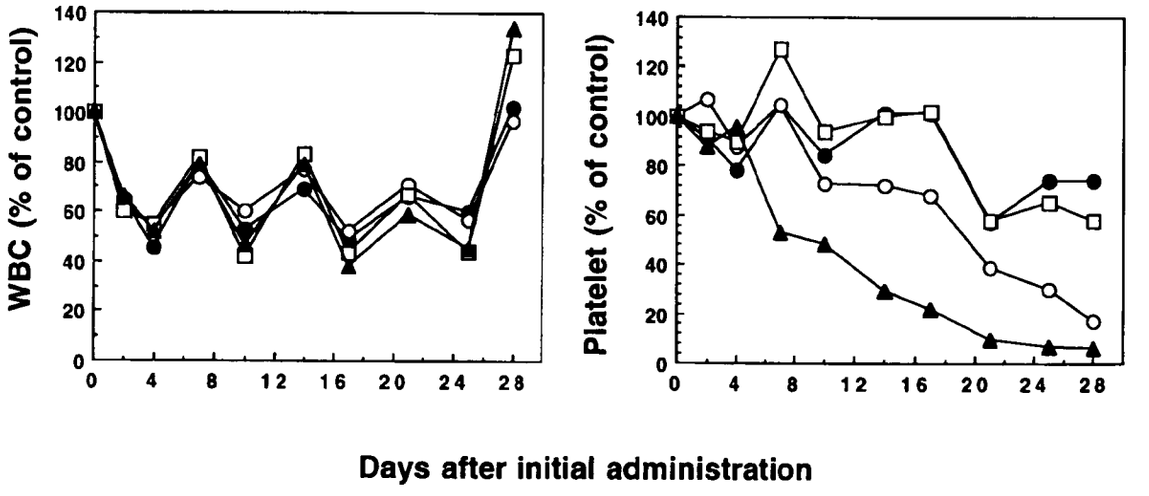

Figure 4. Effect of KW-2149 (●, 17 mg/kg), M-16 (○, 7.9 mg/kg), M-18 (□, 6.7 mg/kg) or MMC (▲, 4.2 mg/kg) on WBC and platelet number. Compounds were injected intravenously on days 0, 7, 14 and 21 in mature female ddY mice.

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