Anti-Cancer Drugs, **4**, pp. 181–188

Reduced bone marrow toxicity of KW-2149, a mitomycin C derivative, in mice

Tadashi Ashizawa, Masami Okabe, Katsushige Gomi and Tadashi Hirata

Pharmaceutical Research Laboratories, Kyowa Hakko Kogyo Co., Ltd, Shimotogari 1188, Nagaizumi-cho, Sunto-gun, Shizuoka-ken 411, Japan. Tel: (+81) 559-89-2007. Fax: (+81) 559-86-7430.

The bone marrow toxicity of KW-2149, a newly synthesized mitomycin C (MMC) derivative, was compared with MMC in different aged ddY mice. Both MMC and KW-2149 exhibited a similar type of leukopenia in young adult mice. However, in mature adult mice, the recovery from leukopenia was rapid in KW-2149-treated mice, whereas in the MMC-treated mice, it was delayed. The difference between KW-2149 and MMC was more significant in thrombocytopenia in mature mice, and that induced by KW-2149 was mild, whereas that by MMC was severe and delayed. This reduced bone marrow toxicity of KW-2149 in mature mice was confirmed by the assay of colony-forming units in culture, progenitors of granulocytes or macrophages, and megakaryocytic colony-forming units, progenitors of platelets, in the bone marrow cells. The bone marrow toxicity and lethal toxicity of MMC was augmented by weekly intermittent treatment; in contrast, that of KW-2149 was not, suggesting that bone marrow toxicity may have a critical role in the lethal toxicity of MMC. The non-cumulative bone marrow toxicity of KW-2149 enabled the weekly intermittent treatment of human lung adenocarcinoma L-27 inoculated into nude mice. Thus its antitumor activity was greater than with single treatment.

Key words: Antitumor, KW-2149, mitomycin C, mouse, toxicity.

Introduction

Mitomycin C (MMC) shows a significant antitumor activity with a broad spectrum against experimental tumors and human neoplastic disease.[1–3] However, its clinical usefulness has been limited primarily by myelosuppression, manifested as leukopenia or thrombocytopenia.[4–6] Therefore new MMC analogs have been extensively investigated to develop a compound with a broader antitumor spectrum and less myelosuppressive activity. One of these analogs is "-N-[2-[[2-(γ-L-glutamylamino)ethyl]-dithio]ethyl]mitomycin C (KW-2149)."

Correspondence to K Gomi

KW-2149 possesses a broad antitumor activity equal or superior to MMC in many experimental tumor systems.[8–12] KW-2149 was effective against MMC-resistant murine leukemia[11,12] and was suggested to be the best analog for use in a clinical trial against lung cancer.[10] The antitumor activity of KW-2149 was more significant by intermittent treatment in nude mice-inoculated tumor systems.[9,11] On the other hand, the bone marrow toxicity, especially thrombocytopenia, of KW-2149 given as a single injection was mild as compared with that of MMC in young adult male mice.[12]

The hematological parameters of mice were reported to vary with their age.[13] The sensitivity of murine bone marrow cells to radiation or MMC also varied with their age, i.e. aged mice were more susceptible to the damage induced by radiation or MMC.[14,15] The present study compared the bone marrow toxicity of KW-2149 and MMC when given as a single or intermittent injection in different aged mice.

Materials and methods

Drugs

MMC and KW-2149 were produced by Kyowa Hakko Kogyo (Tokyo, Japan) and dissolved in sterile 0.9% NaCl solution. Lethal doses (LD_{10}) of MMC and KW-2149 given as an intravenous injection were 4.2 and 16.6 mg kg, respectively, in young adult male ddY mice.[12]

Animals

Young adult male ddY mice (4 weeks old) weighing 19–21 g and mature adult female ddY mice (20–26 weeks old) weighing 40–50 g were obtained from

Japan SLC (Shizuoka, Japan). Young adult male BALB/c-*nu/nu* mice (5 weeks old) weighing 20–25 g were obtained from Clea Japan (Tokyo, Japan). All animal experiments were conducted with five mice in a group.

Hematological toxicity

The peripheral blood was collected from the retroorbital sinus of ddY mice. The number of red blood cells, white blood cells (WBC) or platelets, hematocrit values and hemoglobin concentration were measured by a Toa micro-cell counter CC-180A (Toa Medical Electronics, Hyogo, Japan).

Spleen weight and number of bone marrow cells

Spleens were removed from ddY mice and their wet weight was measured by an electronic balance ER-182A (A & D, Tokyo, Japan). Femurs were collected from ddY mice and bone marrow cells were flushed from the femurs with a 23-gauge needle connected to a 1 ml syringe which was filled with 1 ml of α-MEM (Flow Laboratories, McLean, VA) containing 10% fetal bovine serum (Gibco, Grand Island, NY). The cell suspensions were passed through a mesh (pore size 100) to remove bone fragments and the cell number was counted by a Toa micro-cell counter CC-180A.

Colony forming units in culture (CFU-C) and megakaryocytic colony forming units (CFU-Meg)

The bone marrow cells prepared as described above were suspended in α-MEM containing 10% fetal bovine serum, 10% culture supernatant of poke-weed mitogen-stimulated spleen cells (BALB/c mice) and 0.3% agar (Difco, Detroit, MI) at a concentration of 1 or 5 × 10^4 cells/0.5 ml/well, and seeded on 24-well multidishes (Nunc, Roskilde, Denmark). The cells were incubated at 37°C for 7 days in a humidified atmosphere containing 5% CO_2 in air. Then whole cells were transferred on the glass slides, dried, fixed by 5% glutaraldehyde and stained with acetylcholine esterase for CFU-Meg or Wright–Giemsa for CFU-C.

Antitumor activity

Human lung adenocarcinoma L-27 (8 mm^3 fragment), kindly supplied by Dr Y. Ohnishi, Central Institute of Experimental Animals, Kanagawa, Japan, was inoculated subcutaneously into the flank of nude mice. When the tumor volume was between 100 and 300 mm^3, KW-2149 or MMC was injected intravenously once or intermittently. The length and width of tumors were measured twice a week, and tumor volume was calculated by using the following formula according to the method of the National Cancer Institute:[16]

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

Tumor growth rate was expressed as the mean V'/V'_0 value, where V' is the tumor volume on the day of evaluation and V'_0 is that of the treatment-starting day.

Statistical analysis

The experimental results were analyzed for statistical significance by Mann–Whitney's *U*-test.

Results

Hematological toxicity of KW-2149 or MMC

The hematological toxicity of KW-2149 or MMC was compared between young adult male mice and mature adult female mice (Figure 1). The female breeder mice were used as mature adult mice because they were easily obtained as aged mice. Both KW-2149 and MMC given as a single intravenous injection of LD_{10} decreased the WBC in peripheral blood in young mice (Figure 1A) and mature mice (Figure 1B), and their nadir was on day 4. The degree of leukopenia induced by KW-2149 was almost equivalent to that induced by MMC in young mice, although a slight statistical significance was observed due to small individual variations. However, in mature mice, the leukopenia induced by KW-2149 was mild and reversible as compared with that induced by MMC.

A more significant difference between KW-2149 and MMC was noted in their thrombocytopenic effect (Figure 2). The number of platelets in young

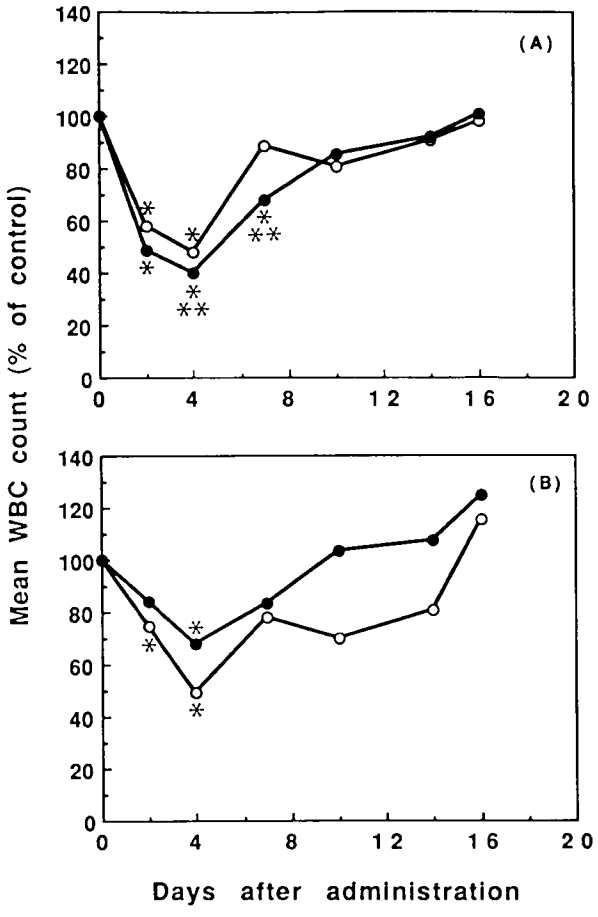

Figure 1. Effect of KW-2149 (●) or MMC (○) on WBC number. KW-2149 (16.6 mg/kg) or MMC (4.2 mg/kg) was injected intravenously once on day 0 in young mice (A) or mature mice (B). $p < 0.05$ versus control group (*) or between KW-2149-treated group and MMC-treated group (**).

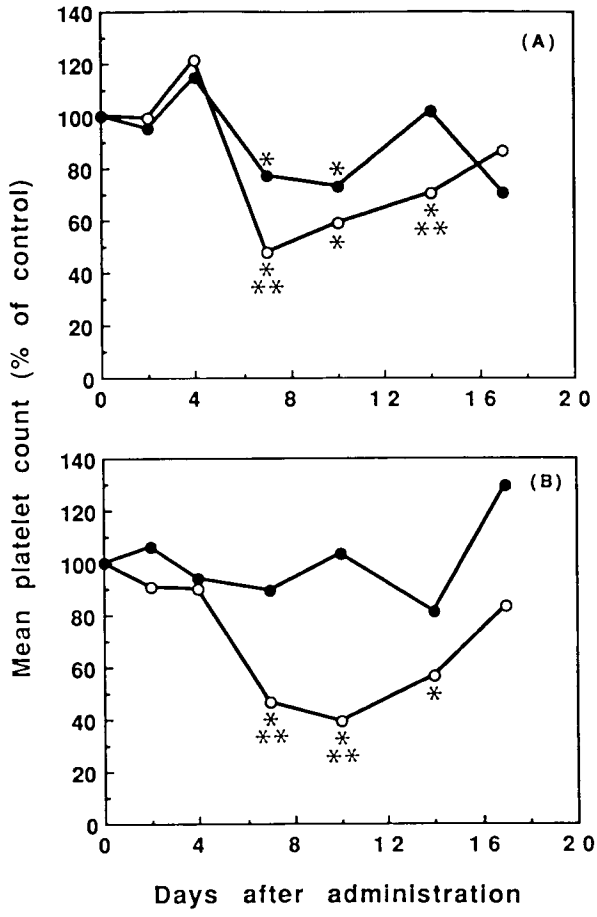

Figure 2. Effect of KW-2149 (●) or MMC (○) on platelet number. KW-2149 (16.6 mg/kg) or MMC (4.2 mg/kg) was injected intravenously once on day 0 in young mice (A) or mature mice (B). $p < 0.05$ versus control group (*) or between KW-2149-treated group and MMC-treated group (**).

mice given MMC as a single injection decreased with a nadir on day 7, and thereafter it recovered gradually (Figure 2A). On the other hand, the decrease of platelet number in mice given KW-2149 was mild. This difference between KW-2149 and MMC was more significant in mature mice (Figure 2B), i.e. the decrease of platelet number by MMC was severe and lasted longer, whereas that of KW-2149 was statistically insignificant.

To examine the reason why hematological toxicity of MMC appeared more severe in mature mice than in young mice, the hematological parameters were compared between young and mature mice (Table 1). Significant differences were not detected in the WBC or platelet counts. The value of hematocrit and hemoglobin was slightly lower in young mice.

The hematological toxicity of KW-2149 and MMC was further compared in mature mice by the intermittent treatment at LD_{10} and two thirds of LD_{10} (Figures 3 and 4). The nadir of WBC was detected after 3 or 4 days from the day of each administration (Figure 3). At two thirds of LD_{10},

Table 1. Hematological parameters of ddY mice

Items	Units	Young mice	Mature mice
White blood cells	$10^2/mm^3$	62 ± 12	67 ± 23
Platelets	$10^4/mm^3$	96 ± 14	114 ± 16
Red blood cells	$10^4/mm^3$	637 ± 28	970 ± 43
Hematocrit	%	52 ± 3	66 ± 4
Hemoglobin	g/dl	12 ± 1	16 ± 1

Mean ± SD.

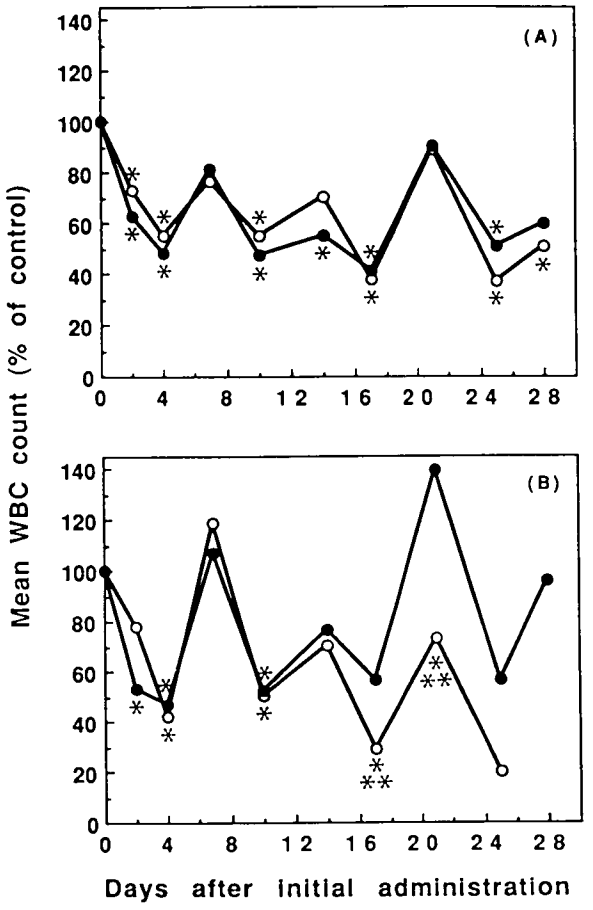

Figure 3. Effect of KW-2149 (●) or MMC (○) on WBC number. KW-2149 [11.1 mg/kg (A) or 16.6 mg/kg (B)] or MMC [2.8 mg/kg (A) or 4.2 mg/kg (B)] was injected intravenously intermittently on days 0, 7, 14 and 21 in mature mice. $p < 0.05$ versus control group (*) or between KW-2149-treated group and MMC-treated group (**).

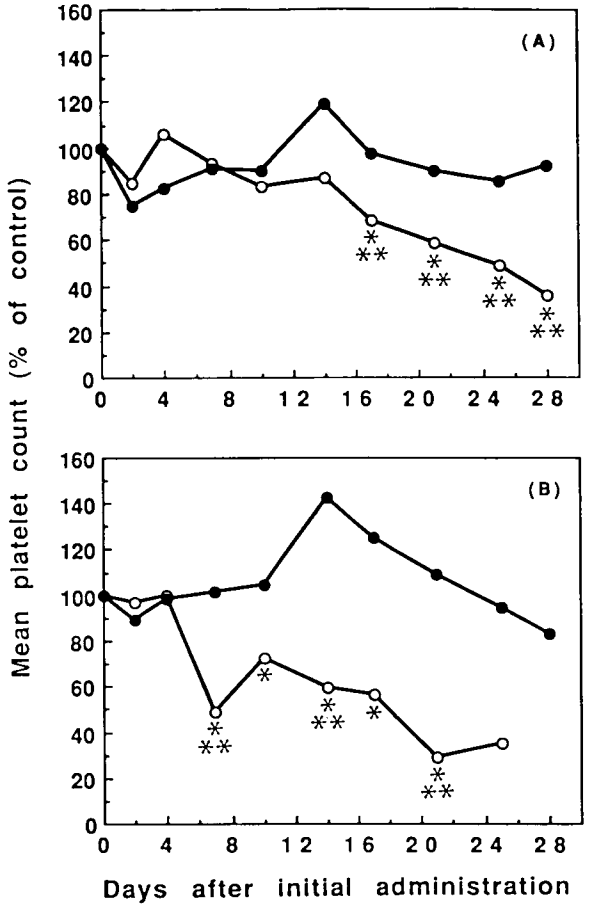

Figure 4. Effect of KW-2149 (●) or MMC (○) on platelet number. KW-2149 [11.1 mg/kg (A) or 16.6 mg/kg (B)] or MMC [2.8 mg/kg (A) or 4.2 mg/kg (B)] was injected intravenously intermittently on days 0, 7, 14 and 21 in mature mice. $p < 0.05$ versus control group (*) or between KW-2149-treated group and MMC-treated group (**).

the difference of leukopenic activity between KW-2149 and MMC was insignificant (Figure 3A). However, at LD_{10}, WBC in mice given KW-2149 recovered almost completely after 1 week (Figure 3B), whereas the cumulative effect of MMC induced leukopenia which did not recover completely. These differences between KW-2149 and MMC were more clearly demonstrated in the peripheral blood platelet counts (Figure 4). At two thirds of LD_{10}, KW-2149 did not cause thrombocytopenia, whereas the gradual and statistically significant decrease of platelet count was detected in MMC-treated mice (Figure 4A). This cumulative effect of MMC was more significant at LD_{10} (Figure 4B).

Effect of KW-2149 or MMC on the hematopoietic stem cells

To clarify the difference of hematological toxicity between KW-2149 and MMC, the effect of both drugs on the hematopoietic progenitor cells was examined (Figure 5). After the administration of KW-2149 and MMC, the weight of spleens decreased to 73 and 57% that of untreated mice, respectively, at nadir on day 4 (Figure 5A), suggesting its association with their hematological toxicity. This decrease of spleen weight by KW-2149 was less than that by MMC. The decrease in the number of bone marrow cells by KW-2149 and MMC was detected earlier (Figure 5B). The

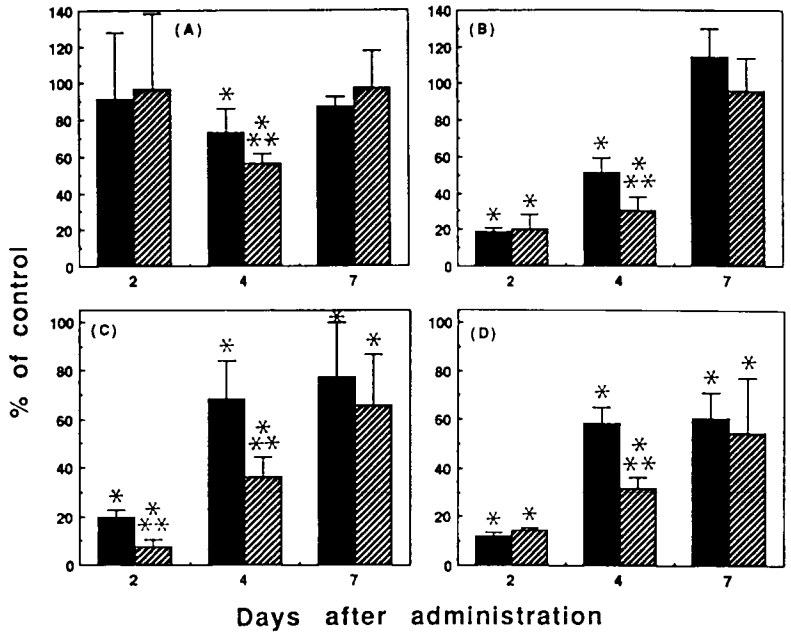

Figure 5. Effect of KW-2149 (solid bar) or MMC (hatched bar) on spleen weight (A), bone marrow cell number (B), CFU-C number (C) and CFU-Meg number (D). KW-2149 (16.6 mg/kg) or MMC (4.2 mg/kg) was injected intravenously once on day 0 in mature mice. Mean ± SD. $p < 0.05$ versus control group (*) or between KW-2149-treated group and MMC-treated group (**).

nadir was on day 2, and the percentages of bone marrow cells in KW-2149- and MMC-treated mice were 19 and 20% of that of the untreated mice, respectively. Thereafter their number recovered rapidly to the normal level on day 7. As seen by the result on day 4, KW-2149-treated mice recovered more rapidly than MMC-treated mice. The reduced toxicity of KW-2149 was supported by the results of its effect on CFU-C, progenitors of granulocytes or macrophages, and CFU-Meg, megakaryocytic progenitors (Figure 5C and D). Both CFU-C and CFU-Meg were decreased by the treatment of KW-2149 or MMC with a nadir on day 2, and then recovered, suggesting that the change of CFU-C or CFU-Meg number was linked to that of bone marrow cell number. The decrease of CFU-C in KW-2149-treated mice was milder than that in MMC-treated mice on day 2, and the recovery was more rapid on days 4 and 7. The recovery of CFU-Meg in KW-2149-treated mice was also more rapid than that in MMC-treated mice on day 4. These results indicate that the reduced hematological toxicity of KW-2149, as shown in Figures 1–4, was explicable by the less toxicity of KW-2149 against bone marrow hematopoietic progenitor cells.

Table 2. Lethal toxicity of KW-2149 or MMC by single or intermittent treatment in young and mature ddY mice

Drugs	Schedule	Dose (mg/kg/day)	Mortality	
			young mice	mature mice
KW-2149	day 0	11.1	0/5	0/5
		16.6	0/5	0/5
MMC	day 0	2.8	0/5	0/5
		4.2	1/5	0/5
KW-2149	days 0, 7, 14 and 21	7.4	0/5	NT
		11.1	0/5	0/5
		16.6	1/5	2/5
MMC	days 0, 7, 14 and 21	1.9	0/5	NT
		2.8	1/5	0/5
		4.2	5/5	5/5

KW-2149 or MMC was injected intravenously on day 0 or days 0, 7, 14 and 21. Survival days were observed for 30 days after final administration. NT = not tested.

Lethal toxicity of KW-2149 and MMC

From the results of Figures 3 and 4, the leukopenia and thrombocytopenia induced by MMC were known to be cumulative, whereas those of

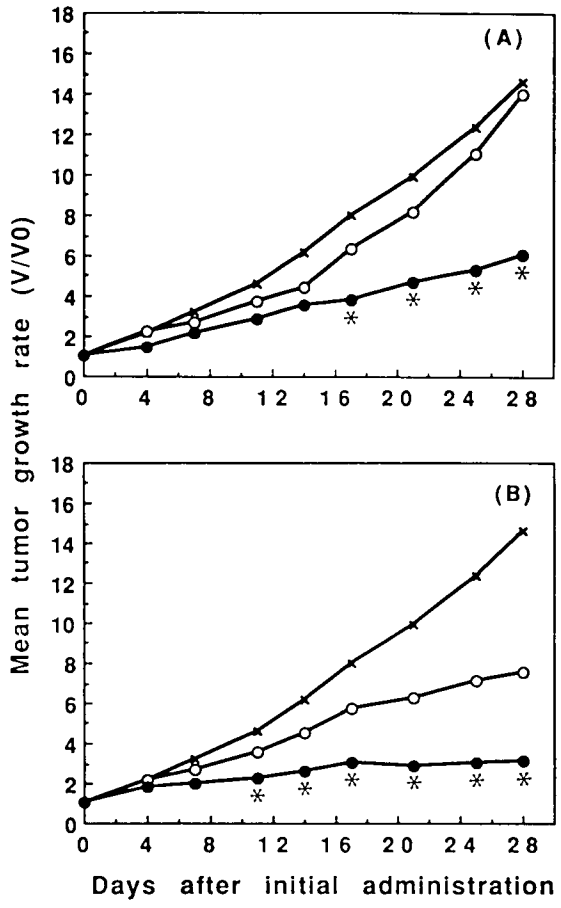

Figure 6. Growth pattern of human lung adenocarcinoma L-27 inoculated into nude mice. KW-2149 (●, 16.6 mg/kg) or MMC (○, 4.2 mg/kg) was injected intravenously on day 0 (A) or days 0, 7, 14 and 21 (B). Control (×). $p < 0.05$ versus control group (*).

KW-2149 were transient. To examine whether these differences of hematological toxicity between both drugs affect their lethal toxicity, the survival days of KW-2149- or MMC-treated mice were observed (Table 2). The intermittent treatment with MMC at LD_{10} on days 0, 7, 14 and 21 augmented its lethal toxicity significantly, suggesting that the accumulation of the hematological toxicity of MMC might be linked to its lethal toxicity. On the other hand, the accumulation of lethal toxicity was not significant in KW-2149-treated mice even after the repeated injections at LD_{10}, suggesting that KW-2149 might be applicable in intensive intermittent treatment.

Antitumor activity of KW-2149 and MMC

From the results in Table 2, the weekly treatment with KW-2149 at LD_{10} was known to be applicable. To examine whether this toxicological characteristic of KW-2149 would result in therapeutic benefits, its antitumor activity was examined against solid tumor of human origin inoculated into nude mice (Figure 6). Human lung adenocarcinoma L-27 was chosen for this purpose, since the antitumor effect of KW-2149 or MMC given as a single injection was moderate against this tumor, as shown in Figure 6(A). As expected, the antitumor activity of both drugs was augmented by the weekly treatment (Figure 6B). The treated versus control values of tumor growth rates on day 28 are 0.96, 0.52, 0.42 and 0.21 in the groups treated with single MMC, intermittent MMC, single KW-2149 and intermittent KW-2149, respectively. The maximum activity of KW-2149 was statistically significant when compared with that of MMC. Considering the results of Figure 6 and Table 2 together, KW-2149 may be expected to show greater antitumor activity by the intermittent treatment regimen.

Discussion

The bone marrow toxicity of MMC was reported to be greater when it was administered by chronic intermittent treatment both experimentally[17] and clinically.[3,6] A large infrequent dose was recommended as an optimal regimen for MMC treatment.[18] Animal models for precise evaluation of bone marrow toxicity of MMC analogs were described,[19] and the mouse CFU-C assay and ferret hematology models were recommended to predict the leukopenia induced by them. Our results indicate that mature female mice are a sensitive model for the evaluation of not only leukopenia but also thrombocytopenia induced by MMC (Figures 1–4).

The difference of hematological toxicity between MMC and KW-2149 was more significant in mature adult female mice than young adult male mice (Figures 1–4). Female breeder mice were used as mature adult mice in these experiments, since they were easily obtained as aged mice. Among two factors, age and sex, the former may be more important, since differences between the sexes were reported to be insignificant in terms of hematological parameters in mice.[13] On the other hand, the age-related changes of bone marrow cells in mice were reported in terms of radiation sensitivity[14] and MMC-induced sister chromatid exchange.[15] In both papers, these changes were explained by a deficiency of bone marrow cells to repair the damage induced by radiation or MMC. The greater bone marrow

toxicity of MMC in mature female mice as shown in Figures 1–4 may be explained by their inability to repair the MMC-induced damage. The results of CFU-C and CFU-Meg assays using MMC-treated bone marrow cells may indicate such damage (Figure 5). The number of CFU-C was already reported to be a useful parameters for the evaluation of hematological toxicity of MMC analogs.[19] Our results on KW-2149 support the usefulness of this assay of CFU-C in predicting the leukopenia induced by MMC analogs. The results of the CFU-Meg assay also indicate its usefulness in predicting the thrombocytopenic activity of MMC analogs as shown in Figures 2 and 4. The slower recovery of CFU-Meg number in MMC-treated mice is indicated to be linked to its cumulative effect on thrombocytopenic activity detected in intermittent treatment (Figure 4). The possible mechanism of reduced bone marrow toxicity of KW-2149 may be explained by its reduced distribution to bone marrow. The hydrophilic property of KW-2149 may affect its tissue distribution. We are now examining this.

The hematological toxicity of MMC was cumulative when given intermittently in mature mice (Figures 3 and 4). This cumulative toxicity of MMC augmented its lethal toxicity (Table 2). On the other hand, the hematological toxicity of KW-2149 was mild as a single dose and did not have the cumulative effect during intermittent treatment (Figures 1–4). Therefore, the lethal toxicity of KW-2149 did not demonstrate the cumulative effect (Table 2); thus, the intensive intermittent treatment of human lung adenocarcinoma L-27 became possible (Figure 6). This permitted an increased total dose, augmenting its antitumor activity. KW-2149 may offer a new regimen of cancer chemotherapy, an intensive intermittent treatment, with less hematological toxicity.

Conclusion

The mature adult female mice were a sensitive and useful model to evaluate the bone marrow toxicity of MMC analogs. In this system, the bone marrow toxicity of KW-2149 was mild and intensive intermittent treatment using KW-2149 became possible

Acknowledgments

We thank Dr Yasuyuki Ohnishi for providing human lung adenocarcinoma L-27, and Mr Makoto Asano, Miss Kumiko Masunaga and Miss Hiroko Takanami for their excellent technical assistance.

References

1. Sugiura K. Studies in a tumor spectrum VIII. The effect of mitomycin C on the growth of a variety of mouse, rat, and hamster tumors. *Cancer Res* 1959; **19**: 438–45.
2. Taguchi T. Mitomycin C in gastric cancer. In: *Mitomycin C– current impact on cancer chemotherapy*. Amsterdam: Excerpta Medica 1982: 51–63.
3. Doll DC, Weiss RB, Issell BF. Mitomycin: ten years after approval for marketing. *J Clin Oncol* 1985; **3**: 276–86.
4. Whittington RM, Close HP. Clinical experience with mitomycin C (NSC-26980). *Cancer Chemother Rep (Part I)* 1970; **54**: 195 8.
5. Godfrey TE. Clinical experience with mitomycin C in large infrequent doses. *Cancer* 1972; **29**: 1647 52.
6. Ogawa M. An overview of mitomycin C studies in Japan. In: *Mitomycin C– current impact on cancer chemotherapy*. Amsterdam: Excerpta Medica 1982; 41–50.
7. Kono M, Saitoh Y, Kasai M, *et al.* Synthesis and antitumor activity of a novel water soluble mitomycin analog; 7-*N*-[2-[[2-(γ-L-glutamylamino)ethyl]dithio]ethyl]mitomycin C. *Chem Pharm Bull (Tokyo)* 1989; **37**: 1128–30.
8. Kubota T, Inada T, Inoue S, *et al.* Antitumor activity of 7-*N*-(2-((2-(γ-L-glutamylamino)ethyl)dithio)ethyl)-mitomycin C (KW-2149) against human tumor xenografts serially transplanted into nude mice. *Jpn J Clin Oncol* 1989; **19**: 216–21.
9. Nishiyama M, Kim R, Jinushi K, *et al.* Antitumor effect of KW-2149, a new mitomycin derivative, administered by different modalities. *In Vivo* 1989; **3**: 375–80.
10. Ohe Y, Nakagawa K, Fujiwara Y, *et al. In vitro* evaluation of the new anticancer agents KT6149, MX-2, SM5887, menogaril, and liblomycin using cisplatin- or adriamycin-resistant human cancer cell lines. *Cancer Res* 1989; **49**: 4098–102.
11. Tsuruo T, Sudo Y, Asami N, *et al.* 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*. *Cancer Chemother Pharmacol* 1990; **27**: 89–93.
12. Morimoto M, Ashizawa T, Ohno H, *et al.* Antitumor activity of 7-*N*-{{2-{[2-(γ-L-glutamylamino)ethyl]dithio}-ethyl}}mitomycin C. *Cancer Res* 1991; **51**: 110–5.
13. Wolford ST, Schroer RA, Gohs FX, *et al.* Reference range data base for serum chemistry and hematology values in laboratory animals. *J Toxicol Environm Health* 1986; **18**: 161–88.
14. Chen MG. Age-related changes in hematopoietic stem cell populations of a long-lived hybrid mouse. *J Cell Physiol* 1971; **78**: 225–32.
15. Kram D, Schneider EL, Tice RR, *et al.* Aging and sister chromatid exchange. I. The effect of aging on mitomycin-C induced sister chromatid exchange frequencies in mouse and rat bone marrow cells *in vivo*. *Exp Cell Res* 1978; **114**: 471–5.
16. Geran RI, Greenberg NH, MacDonald MM, *et al.* Protocols for screening chemical agents and natural products against animal tumors and other biological systems. *Cancer Chemother Rep Part III* 1972; **3**: 1–103.

17. Rockwell S, Nierenburg M, Irvin CG. Effects of the mode of administration of mitomycin on tumor and marrow response and on the therapeutic ratio. *Cancer Treat Rep* 1987; **71**: 927–34.
18. Taguchi T. Advances in anticancer drugs in the past decade: progress in mitomycin study. In: *New trends in cancer chemotherapy with mitomycin C*. Amsterdam: Excerpta Medica 1987; 1–24.
19. Schurig JE, Schlein A, Florczyk AP, *et al.* Animal models for evaluating the myelosuppressive effects of cancer chemotherapeutic agents. *Exp Hematol* 1985; **13** (Suppl 16): 101–5.

(Received 25 January 1993; accepted 3 February 1993)