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Comparison of uptake of mitomycin C and KW-2149 by murine P388 leukemia cells sensitive or resistant to mitomycin C

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Summary. KW-2149, a new mitomycin C (MMC) derivative, inhibited the growth of murine P388 leukemia in vitro at 20-fold lower concentrations than those of MMC. KW-2149 was also effective in inhibiting the growth of MMC-resistant P388 (P388/MMC) cells. To elucidate these characteristics of KW-2149, its uptake and efflux were compared with those of MMC in MMC-sensitive and -resistant P388 cells. Both MMC and KW-2149 accumulated rapidly in P388 cells after incubation at the concentration of 0.47 and 0.024 μM, respectively, which were the IC_{50} values at 1-h exposure. Although this concentration of KW-2149 was 20 times lower than that of MMC, its intracellular concentration was little more than that of MMC, suggesting that KW-2149 accumulated in the cells quite efficiently. The accumulated KW-2149 in the cells after 1-h treatment remained for as long as 24 h after the incubation of the cells in drug-free medium, suggesting that most of the intracellular KW-2149 or MMC was bound to cellular components. The ratios of resistance of P388/MMC cells to MMC and KW-2149 were 34 and 8.8, respectively, at 1-h exposure, suggesting that P388/MMC cells were partially resistant to KW-2149 in vitro. P388/MMC cells also showed partial resistance to cisplatin, Adriamycin, *m*-AMSA, and etoposide. The accumulation of MMC in P388/MMC cells was lower than that in P388 cells, although the size of the former cells was almost equal to that of the latter. As a result, the amount of DNA-bound MMC was lower in P388/MMC cells than in P388 cells, suggesting its involvement in the mechanisms of MMC resistance in P388/MMC cells.

Introduction

7-*N*-{{2-{[2-(γ-L-Glutamylamino)ethyl]dithio}ethyl}}-mitomycin C (KW-2149) is a newly synthesized water-soluble derivative of MMC (Fig. 1) [7]. KW-2149 possesses broad antitumor activity equal or superior to that of MMC in many experimental tumor systems [8, 11–13, 17]. The in vitro growth-inhibitory activity of KW-2149 against human tumor cell lines was 10- to 100-fold that of MMC [11]. KW-2149 was also effective in inhibiting the growth of MMC-insensitive human tumor cell lines in vitro [11]. One of the remarkable features of the in vivo antitumor activity of KW-2149 was its effectiveness against MMC-resistant murine leukemia P388 and L1210 cells [11, 17], suggesting that KW-2149 might possess a mode of action somewhat different from that of MMC. However, the mechanisms of such effectiveness remain undetermined.

For the study of drug disposition at the cellular level, the use of labeled compounds seems to be essential. However, labeled MMC with the appropriate specific radioactivity and chemical stability for this purpose did not exist. Recently, the chemical modification of the C6-methyl position of mitomycins was achieved [6]. This enabled the synthesis of chemically stable labeled compounds of MMC such as [C6-CH_3-^{14}C]- and [C6-CH_3-$^3H_3$]-MMC with a high specific radioactivity [1].

In the present study, the mechanisms of the effectiveness of KW-2149 against murine MMC-sensitive and -resistant P388 leukemia were investigated using the labeled compounds described above.

Materials and methods

Chemicals. MMC and KW-2149 (both produced by Kyowa Hakko Kogyo Co., Tokyo, Japan) were dissolved in sterile 0.9% NaCl solution. [C6-CH_3-$^3H_3$]-MMC (78.4 Ci/mmol) was synthesized by Amersham International (Buckinghamshire, UK) [1, 6]. [C6-CH_3-$^3H_3$]-KW-2149 (40 Ci/mmol) was synthesized from [C6-CH_3-$^3H_3$]-mitomycin A according to the method described in the literature [7]. These labeled

Abbreviations: *m*-AMSA, 4′-(9-acridinylamino)methanesulfon-*m*-aniside; HPLC, high-performance liquid chromatography; IC_{50}, concentration required for 50% growth inhibition; MMC, mitomycin C; PFM, porfiromycin

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compounds were dissolved in methanol and used after dilution with unlabeled compounds to the designated concentration.

Cisplatin (Sigma Chemical Co., St. Louis, Mo.), Adriamycin (Kyowa Hakko Kogyo Co.), etoposide (Nippon Kayaku Co., Tokyo, Japan), bleomycin (Nippon Kayaku Co.), and 5-fluorouracil (Kyowa Hakko Kogyo Co.) were dissolved in sterile distilled water. *m*-AMSA, kindly supplied by the Drug Evaluation Branch, Developmental Therapeutics Program, National Cancer Institute, was dissolved in dimethyl sulfoxide. All compounds were diluted with the culture medium, and the final concentration of solvent was 0.5% and less.

Tumor cells. The P388 murine lymphocytic leukemia cell line and its MMC-resistant subline P388/MMC [5] were passaged in vivo in male adult DBA/2 mice (Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka, Japan) weighing 20–25 g. These cell lines were passaged in vitro at least five times in RPMI-1640 medium (Grand Island Biological Co., Grand Island, N. Y.) containing 10% fetal bovine serum (Grand Island Biological Co.), 100 IU penicillin, 100 μg streptomycin/ml (Grand Island Biological Co.), and 20 μM 2-mercaptoethanol (Wako Pure Chemical Co., Osaka, Japan), hereafter designated as the culture medium, before the experiments. The sensitivity of both cell lines to MMC and KW-2149 was stable for at least 20 in vitro passages (data not shown).

Cell growth-inhibitory activity. The cells (2×10^3/well) were precultured for 24 h in 96-well microplates (Nunc, Roskilde, Denmark) with 0.1 ml culture medium in each well at 37° C in a humidified atmosphere containing 5% CO_2 in air. The cells were then treated with 0.05 ml drug solution for 1 or 72 h. The growth-inhibitory activity of drugs was evaluated by counting the number of cells using a Micro-cell counter (Toa Medical Electronics Co., Hyogo, Japan).

Analysis of cell size. The cells (2×10^4/ml) were precultured for 24 h, washed, and suspended in Dulbecco's phosphate-buffered saline (Ca^{2+}- and Mg^{2+}-free). The size of cells was analyzed with an Automatic Particle Size Analyzer (HIAC Instrument Division of Pacific Scientific Company, Montclair, Calif.) at 1×10^3 cells/ml.

Uptake of labeled compounds. The cells (1×10^6/well) were precultured for 24 h in 24-well multidishes (Nunc) containing 0.45 ml culture medium in each well at 37° C. The cells were then treated with 0.05 ml drug solution, incubated for the indicated time, transferred onto GF/C glass microfiber filters (Whatman International, Maidstone, UK), and washed with 0.9% NaCl solution for 2 min. The radioactivity of the dried glass filter was measured in a liquid scintillation counter. For measuring the incorporation of labeled compounds into nuclei, the cells were lysed with 0.5% NP-40 solution (Sigma Chemical Co.) at 0° C for 10 min, washed with 0.9% NaCl solution, and centrifuged. The pellets were lysed with 1 *N* NaOH solution at 37° C overnight, and their radioactivity was measured.

Efflux of labeled compounds. The cells (7.5×10^6/6.75 ml) were precultured in plastic flasks (Nunc) for 24 h as described above, treated with 0.75 ml drug solution, and incubated at 37° C for 60 min. They were then centrifuged, washed with cold RPMI-1640 medium, resuspended in culture medium, and incubated in 24-well multidishes at 37° C for the indicated time. The radioactivity of cells was measured as described above.

DNA extraction. The extraction of DNA from cells was performed as previously described [9], with minor modifications. Briefly, the cells (5×10^7/50 ml) were precultured in plastic flasks (Nunc). After 24 h, the cells were treated with [^{3}H]-MMC at 37° C. At each time indicated, the cells (5 ml) were removed from the flasks, washed with sterile 0.9% NaCl solution, suspended in 200 mM disodium ethylenediaminetetraacetic acid (EDTA, pH 8.0), and treated with 1 mg proteinase K/ml (Boehringer-Mannheim Yamanouchi Co., Tokyo, Japan) and 1% Sarkosyl at 47° C for 4 h under occasional shaking. DNA was purified by phenol extraction and ethanol precipitation, and its grade was assessed by the ratio of its absorbance at 260 and 280 nm over 1.8. One optical density unit at 260 nm was calculated to be 50 μg DNA/ml.

Results

Growth-inhibitory activity of KW-2149 or MMC against P388 and P388/MMC cells

We have previously reported that KW-2149 increases the life span of MMC-resistant P388 leukemia-bearing mice as well as parent MMC-sensitive P388 leukemia-bearing mice [12]. To learn the mechanisms of this effectiveness of KW-2149, we first examined the sensitivity of P388 cells and P388/MMC cells to MMC and KW-2149 in vitro (Table 1). The IC_{50} value determined for KW-2149 against sensitive P388 cells at 1-h exposure was 20 times lower than that found for MMC, indicating that the growth-inhibitory activity of KW-2149 was 20-fold that of MMC. Both compounds inhibited the growth of P388 cells at lower concentrations at 72-h exposure, suggesting that the activity of both compounds was considerably stable in the culture medium. P388/MMC cells exhibited a resistance ratio of 34 against MMC at 1-h exposure, suggesting that this cell line acquired MMC resistance. On the other hand, the resistance ratio of P388/MMC cells against KW-2149 was 8.8 at 1-h exposure, suggesting that this cell line showed partial resistance to KW-2149 in vitro.

For further characterization of P388/MMC cells, their sensitivity to various chemotherapeutic drugs was compared with that of P388 cells (Table 2). P388/MMC cells showed ratios of resistance of about 3 to cisplatin, Adria-

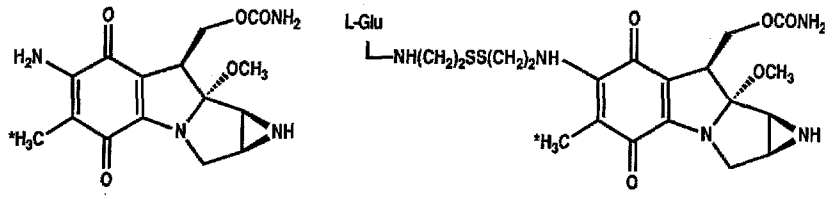
Table 1. Growth-inhibiting activity of MMC and KW-2149 against P388 leukemia sensitive or resistant to MMC

Drugs	Exposure time					
	1 h			72 h		
	IC_{50} (μM)[a]		Resistance ratio[b]	IC_{50} (μM)		Resistance ratio
	P388	P388/MMC		P388	P388/MMC	
MMC	0.47	16	34	0.013	0.47	36
KW-2149	0.024	0.21	8.8	0.0017	0.014	8.2

P388 or P388/MMC cells (2×10^3/well) were cultured on day 0 and treated with MMC or KW-2149 on day 1 for 1 or 72 h. On day 4, the growth-inhibiting activity was determined as described

[a] Mean value for 3 representative experiments

[b] Ratio of IC_{50} values for P388/MMC cells to those for P388 cells, respectively



[C6-CH3-3H3]mitomycin C [C6-CH3-3H3]KW-2149

Fig. 1. Structures of [^{3}H]-MMC and [^{3}H]-KW-2149. The pentagrams show the position of tritium

Table 2. Sensitivity of P388 and P388/MMC cells to other antitumor drugs

Drugs	IC_{50} (μM)		Resistance ratio[a]
	P388	P388/MMC	
Cisplatin	0.32	1.1	3.4
Adriamycin	0.011	0.033	3.0
m-AMSA	0.0076	0.061	8.0
Etoposide	0.025	0.091	3.6
Bleomycin	>50	>50	–
5-Fluorouracil	0.67	0.62	0.93

P388 or P388/MMC cells (2×10^3/well) were cultured on day 0 and treated with each drug on day 1 for 72 h. On day 4, the growth-inhibitory activity was determined

[a] Ratio of IC_{50} values for P388/MMC cells to those for P388 cells, respectively

Table 3. Accumulation of MMC or KW-2149 into P388 cells at their IC_{50}

Drugs	IC_{50} (μM)	Accumulation (pmol/10^6 cells)	
		4 h	8 h
MMC	0.47	0.41	0.57
KW-2149	0.024	0.50	0.75

The data used for Fig. 2 are shown

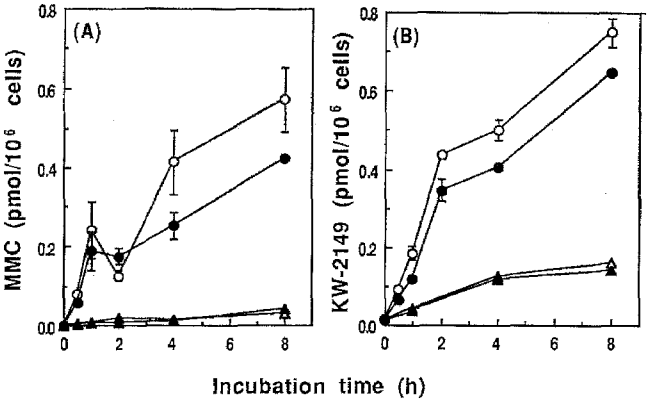


Fig. 2A, B. Uptake of MMC or KW-2149 in P388 or P388/MMC cells and their accumulation in nuclei. P388 (○, △) or P388/MMC (●, ▲) cells (1×10^6/well) were cultured on day 0 and treated with **A** 0.47 μM [^{3}H]-MMC or **B** 0.024 μM [^{3}H]-KW-2149 on day 1 for the indicated time. The radioactivity of cells (○, ●) and nuclei (△, ▲) was measured, and mean values ± SD are shown

mycin, *m*-AMSA, and etoposide, although these were lower than that against MMC. P388/MMC cells did not show cross-resistance to 5-fluorouracil at all.

Uptake of KW-2149 or MMC

The uptake of KW-2149 in P388 cells and P388/MMC cells was compared with that of MMC (Fig. 2). The IC_{50} values found for KW-2149 and MMC at 1-h exposure against P388 cells (0.024 and 0.47 μM, respectively) were used for both cell lines in this experiment. Both MMC (Fig. 2A) and KW-2149 (Fig. 2B) accumulated rapidly in P388 and P388/MMC cells. After 8 h, the amounts of MMC in P388 and P388/MMC cells were 0.57 and 0.42 pmol/10^6 cells, respectively, and the amounts of KW-2149 were 0.75 and 0.65 pmol/10^6 cells, respectively. Although the concentration of KW-2149 in the culture medium was 20 times lower than that of MMC, its uptake in P388 cells was little more than that of MMC (Table 3), suggesting that the uptake of KW-2149 in the cells was extremely efficient. At 8 h after drug treatment, the amount of MMC detected in the nuclei of P388 cells was 5.5% of its total intracellular radioactivity and the amount of KW-2149 was 22%, suggesting that KW-2149 accumulated in the nuclei more rapidly.

The uptake of MMC by P388/MMC cells was slightly but apparently lower than that by P388 cells at the incubation time of 4 or 8 h. Since the size of P388/MMC cells was almost equal to that of P388 cells (data not shown), decreased MMC accumulation may correlate with resistance in the P388/MMC cells.

Efflux of KW-2149 or MMC

The retention of sufficient intracellular drug concentration was necessary for the effective killing of tumor cells. Therefore, the efflux of KW-2149 and MMC was examined in both P388 and P388/MMC cells (Fig. 3). Most of the MMC or KW-2149 molecules that had accumulated in both cell lines during the 1-h treatment were retained within the cells for as long as 24 h after the incubation of cells in drug-free medium, suggesting that most of the intracellular KW-2149 or MMC was bound to cellular components.

Reaction of MMC with DNA

To investigate the mechanisms of MMC resistance in P388/MMC cells further, DNA was extracted and purified

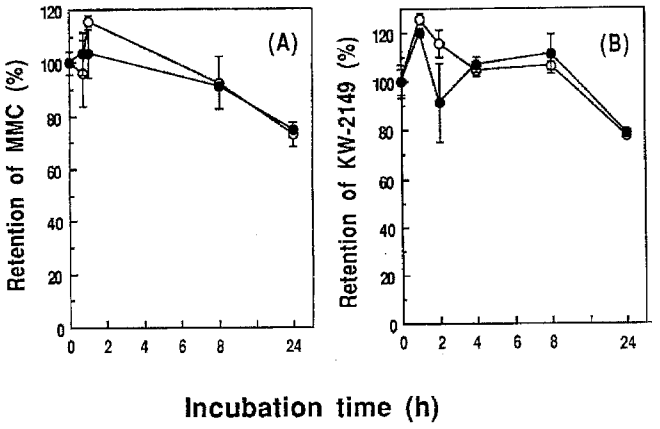


Fig. 3 A, B. Efflux of MMC or KW-2149 from P388 or P388/MMC cells. P388 (○) or P388/MMC (●) cells (7.5×10^6/flask) were cultured on day 0 and treated with **A** 0.47 μM [^{3}H]-MMC or **B** 0.024 μM [^{3}H]-KW-2149 on day 1 for 60 min. The cells were then washed and further incubated in drug-free medium for the indicated time, and the radioactivity of the cells was measured. Mean values ±SD are shown

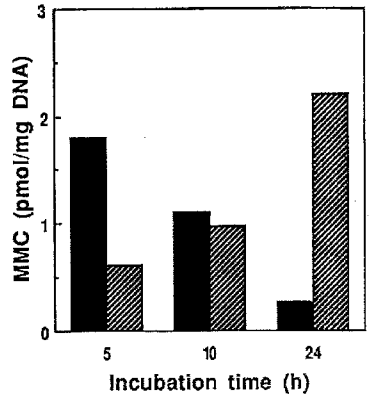


Fig. 4. Binding of MMC to DNA of P388 or P388/MMC cells. P388 (*black bars*) or P388/MMC (*hatched bars*) cells were cultured on day 0 and treated with 0.47 μM [^{3}H]-MMC on day 1 for the indicated time. The extraction of DNA from cells is described in Materials and methods

from MMC-treated P388 or P388/MMC cells, and the amount of DNA-bound MMC was compared (Fig. 4). MMC was not detected in DNA after 1-h incubation (data not shown). However, after 5 h, 1.8 and 0.61 pmol MMC was detected in 1 mg DNA of P388 cells and P388/MMC cells, respectively. Subsequently, the amount of DNA-bound MMC in P388 cells decreased until 24 h, whereas that in P388/MMC cells increased. The amount of DNA-bound MMC in P388/MMC cells after 24 h was almost equivalent to that in P388 cells after 5 h. These results may be explained by the concentration of MMC used in this experiment, namely, the IC_{50} at 1-h exposure against P388 cells. Treatment of P388 cells at this concentration for the longer exposure time might decrease their viability significantly, as is suggested from the results of 72-h exposure in Table 1. As a result, the decrease in DNA-bound MMC might occur through unknown mechanisms. On the other hand, the MMC concentration (0.47 μM) in P388/MMC cells corresponded to the IC_{50} value at the 72-h exposure and was not found to be lethal at the exposure time of 5 or 24 h. Therefore, the amount of DNA-bound MMC in viable P388/MMC cells seemed to have increased until 24 h. The IC_{50} value found for MMC against P388/MMC cells at 24-h exposure (data not shown) was almost the same as that determined for MMC against P388 cells at 1-h exposure. These results indicate that the reduced amount of DNA-bound MMC in P388/MMC cells might be involved in the mechanisms of MMC resistance.

Discussion

Concerning the cellular disposition of MMC analogs, several papers have been published [2, 3, 10, 14–16, 18]. However, the labeled compounds used were porfiromycin (PFM), of which the 1a position was relatively susceptible to labeling with tritium or carbon 14 [3, 10, 14–16], and the disposition of MMC was analyzed only by high-performance liquid chromatography (HPLC) [2, 18], presumably because of the difficulty in synthesizing the chemically stable labeled compound. As for the results obtained using labeled PFM, the different characteristics of cytotoxicity between PFM and MMC [4] should be considered to interpret its cellular disposition. Furthermore, the labeled tritium or carbon 14 at the methyl moiety of position 1a of PFM might be metabolized through its bioactivation in the cells [16]. A considerably high concentration of MMC was used for HPLC analysis because of the low detection limit of MMC, suggesting that the cellular disposition of MMC at a therapeutically rational concentration remains undetermined. Therefore, chemically stable labeled MMC is expected to be useful in future studies.

The uptake of MMC in P388 cells at its IC_{50} value at 1-h exposure (0.47 μM) was 0.41 pmol/10^6 cells after 4 h (Table 3). This amount of MMC was coincidental with the result obtained by Wallner et al. [18] using labeled PFM. On the other hand, Dorr et al. [2] reported the accumulation in murine leukemia L1210 cells of amounts of MMC larger than those found in our studies. This disagreement might be explained by the high concentration of MMC (300 μM) used by the latter authors for HPLC analysis [2]. Interestingly, the accumulation of KW-2149 was almost equal to that of MMC in P388 cells, although the concentration of KW-2149 in the culture medium was about 20 times lower than that of MMC (Table 3), suggesting that the uptake of KW-2149 in P388 cells was quite efficient as compared with MMC uptake. We further confirmed that the uptake of both MMC and KW-2149 into P388 cells increased linearly for 60 min after treatment at a concentration below 10 μM, and the amount of KW-2149 taken up was about 20-fold that of MMC at an equimolar concentration (data not shown). The potent growth-inhibiting activity of KW-2149 against P388 cells, as shown in Table 1, might be explained by its efficient uptake in the cells. We have previously reported that the growth-inhibiting activity of KW-2149 against cultured human tumor cell lines is 10- to 100-fold that of MMC [11]. This activity of KW-2149 might also be explained by its efficient uptake in these human tumor cell lines.

The efflux of MMC was very slow in both P388 and P388/MMC cells (Fig. 3). Since the modality of transport of PFM from the medium into the cells has been reported to be passive diffusion, whereby the specific carrier proteins were not indicated [15], the transport of MMC may also occur via passive diffusion. The significantly low level of efflux of MMC may be explained by the rapid biochemical activation of intracellular MMC and its subsequent reaction with DNA or other intracellular macromolecules. Although the efflux of MMC has been reported in Chinese hamster ovary cells by HPLC analysis [18], the disposition of unreacted MMC was only suggested to have been detected by this method.

The mechanisms of MMC resistance have been investigated in terms of the transport of MMC or PFM [2, 3, 10, 18]. Little difference was found in the transport of MMC to MMC-sensitive and -resistant cells in some cases [3, 18], whereas a partial decrease in the accumulation of MMC was reported in murine L1210 leukemia [2]. Our result is coincident with the latter case; namely, a partial decrease in MMC accumulation was detected in P388/MMC cells as compared with P388 cells (Fig. 2). This reduced accumulation of MMC in P388/MMC cells might lead to the reduced amount of DNA-bound MMC (Fig. 4), suggesting an association with the mechanisms of MMC resistance in P388/MMC cells to a certain extent. The experiments on DNA cross-linking induced by MMC and KW-2149 will provide evidence in substantiating the role of differences in cellular pharmacokinetics and DNA levels between MMC and KW-2149 as determinants of resistance. On the other hand, the efflux of MMC from P388/MMC cells was insignificant, and its pattern was similar to that of MMC from P388 cells (Fig. 3). We have confirmed that the levels of P-glycoprotein expression in P388/MMC cells are similar to those in P388 cells by immunoblotting (data not shown). These results suggest that P-glycoproteins might not be involved in the mechanisms of resistance of P388/MMC cells to MMC.

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