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Comparative studies on the action of 7-*N*-[2-[[2-(γ-L-glutamylamino)ethyl]dithio]ethyl]mitomycin C and of mitomycin C on cultured HL-60 cells and isolated phage and plasmid DNA

Chikashi Ishioka, Ryunosuke Kanamaru, Yukari Konishi, Akira Ishikawa, Hiroyuki Shibata, and Akira Wakui

Department of Clinical Cancer Chemotherapy, The Research Institute for Tuberculosis and Cancer, Tohoku University, 4-1, Seiryo-machi, Sendai 980, Japan

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Summary. The mechanism of action of a new mitomycin C (MMC) derivative, KT6149, was studied in human leukemia HL-60 cells and in isolated phage and plasmid DNA, and its effects were compared with those of MMC. Cell growth was markedly inhibited by KT6149, with an IC_{50} of 2×10^{-9} *M*, that for MMC being 2×10^{-8} *M*. DNA synthesis of HL-60 cells as determined by incorporation of [^{3}H]-thymidine was also inhibited by KT6149, with an IC_{50} of 2×10^{-7} *M* as compared with 2×10^{-6} *M* for MMC. RNA and protein synthesis were less markedly inhibited at low concentrations. Alkaline sucrose density-gradient centrifugation revealed a significant decrease in sedimentation velocity for cellular DNA of the cells after 1 h treatment with KT6149 at concentrations higher than 10^{-7} *M*. In contrast, no such change was observed for DNA of cells treated with MMC, even at a concentration of 10^{-5} *M*. In a cell-free system, analysis by agarose gel electrophoresis patterns showed that the drug induced a decrease in the amount of covalently closed circular DNA of phage PM2 and an increase in that of open circular DNA in the presence of dithiothreitol (DTT), whereas MMC did not cause any change in DNA subfraction amounts. Furthermore, the electrophoretic mobility of linearized pBR322 DNA in alkaline agarose gel was significantly decreased by KT6149 in the presence of DTT and $FeSO_4$, no such change being observed in the case of MMC. The results clearly indicate that the inhibitory effects of KT6149 on the growth and DNA synthesis of HL-60 cells are more potent than those of MMC and that KT6149-induced DNA damage is due to single-strand scission and to cross-linking of DNA, suggesting a mode of activation different from that of MMC.

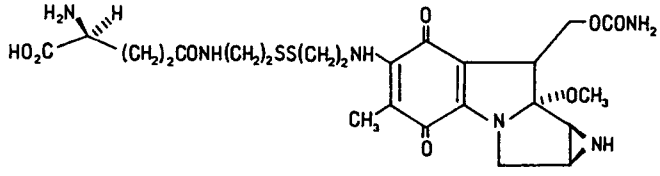

Fig. 1. Chemical structure of KT6149

Introduction

The novel antitumor antibiotic 7-*N*-[2-[[2-(γ-L-glutamylamino)ethyl]dithio]ethyl]mitomycin C (KT6149), which is a synthetic derivative of mitomycin C (MMC), is produced by Kyowa Hakko Kogyo Co. Ltd. This compound is dark blue and crystalline, with a molecular weight of 598.70, and is water-soluble and stable in aqueous solution. Its chemical structure is shown in Fig. 1.

Previous studies have shown that KT6149 exhibits potent antitumor activity against a variety of animal neoplasms and human tumor xenografts and has a strong cytotoxic effect against various cultured tumor cells in vitro [3]. In addition, this drug also shows activity against leukemias P388 and L1210, which are refractory to MMC. However, the mechanisms by which KT6149 induces these antitumor and cytotoxic effects have not yet been defined.

A number of anticancer drugs are known to induce DNA lesions, and it has previously been clarified that the ultimate effects of MMC on DNA are alkylation and single-strand scission [5]. In the present study, the cytotoxic activity and the mode of action of the MMC derivative KT6149 were investigated in human leukemia HL-60 cells and in isolated phage and plasmid DNA and the effects were compared with those of MMC.

Materials and methods

Chemicals. KT6149 and MMC were generously supplied by Kyowa Hakko Co. Ltd., Tokyo, Japan. [6-^{3}H]-thymidine (19.3 Ci/mmol), [6-^{3}H]-uridine (4.2 Ci/mmol), L-[3,4,5-^{3}H]-leucine (147.0 Ci/mmol),

Abbreviations: KT6149, 7-N-[2-[[2-(γ-L-glutamylamino)ethyl]dithio]-ethyl]mitomycin C; MMC, mitomycin C; IC_{50}, concentration of drug that inhibits cell growth or incorporation of radiolabeled precursors by 50%; DTT, dithiothreitol

Offprint requests to: C. Ishioka

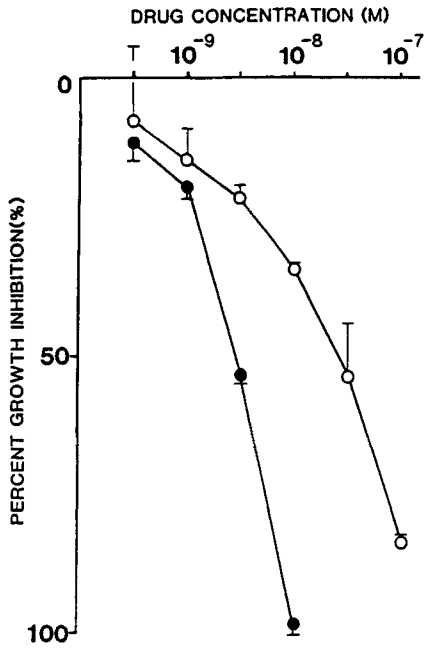

Fig. 2. Effects of KT6149 on the growth of HL-60 cells. The numbers of viable cells at culture day 3 were used to calculate the percentage of growth inhibition as described in the text. O——O, MMC; ●——●, KT6149. Values represent the mean ± SD (bars)

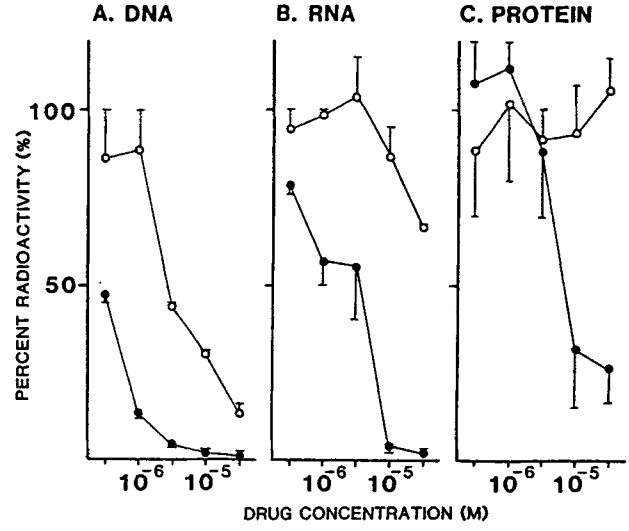

Fig. 3 A–C. Effect of KT6149 on A DNA, B RNA, and C protein synthesis of HL-60 cells. DNA, RNA, or protein synthesis was assessed by the incorporation of specific radioactive precursors into the acid-insoluble fraction of the cells as described in the text. The percentage of control radioactivity was calculated from the observed radioactivity values. O——O, MMC; ●——●, KT6149. Values represent the mean ± SD (bars)

and [2-^{14}C]-thymidine (52.0 Ci/mmol) were obtained from New England Nuclear. All other chemicals were of biochemical reagent grade.

Cells and cell culture. Human promyelocytic leukemia cell line HL-60 was kindly provided by Dr. H. Hemmi (Tohoku University, Sendai, Japan). HL-60 cells were suspended in RPMI 1640 (Nissui Seiyaku Co. Ltd., Tokyo, Japan) supplemented with 10% heat-inactivated (56° C, 30 min) fetal calf serum (Gibco Laboratories, Chagrin Falls, Ohio), penicillin (50 IU/ml), and kanamycin (50 μg/ml). Cultures were incubated at 37° C in a water-saturated atmosphere containing 5% CO_2.

The cells were seeded at 2.5×10^5/ml in a total volume of 10 ml in 50-ml 25-cm^2 culture flasks (Falcon 3013, Becton Dickinson, Oxnard, Calif) and cultured in the presence of different concentrations of KT6149 or MMC. Viable cells were counted by the trypan blue exclusion test in a hemocytometer. The number of viable cells from each culture was used to calculate the percentage of growth inhibition according to the formula $100 \times [(Nc\text{-}Ne)/(Nc\text{-}No)]$, where Nc is the number of cells counted in control culture, Ne is the number of cells in KT6149- or MMC-treated culture, and No is the number of cells in the initial culture.

Measurement of macromolecular synthesis of HL-60 cells. HL-60 cells (2×10^5/ml, 2 ml) were cultured in the presence or absence of KT6149 or MMC for 4 h at 37° C. [^{3}H]-thymidine, [^{3}H]-uridine, or [^{3}H]-leucine were added to each culture to give a final concentration of 0.5 μCi/ml, and the cultures were further incubated for 1 h. The cells were washed and resuspended in 5 ml PBS, then trapped on glass-fiber filter paper (Whatman GF/C 24 mm) and washed with 15 ml PBS, 30 ml ice-cold trichloroacetic acid (TCA), and 15 ml 95% ethanol, successively. After drying, glass-fiber filter radioactivity was counted in scintillation fluid (2,5-diphenyl-oxazol, 5 g; toluene, 1 l) with a liquid scintillation spectrometer (Beckman model LS 250).

Alkaline sucrose density-gradient centrifugation analysis of DNA. HL-60 were treated with [^{14}C]-thymidine (0.1 μCi/ml) for 24 h, followed by incubation in the absence of radiolabeled compound for 24 h. The cells were then subcultured and treated with KT6149 or MMC for 1 h. Using polyallomer tubing, 13 ml of linear gradients of 5%–20% sucrose containing 0.3 *N* NaOH, 0.7 *M* NaCl, and 10 m*M* Na_2-EDTA were formed and 1.5 ml lysing solution containing 0.55 *N* NaOH, 0.45 *M* NaCl, 10 m*M* Na_2-EDTA, and 0.015% Sarcosyl was layered on the top of each gradient. Then, 0.1 ml PBS containing 3×10^4 cells was layered gently onto the lysing solution. Lysis was carried out for 5 h at 4° C in a dark environment. The gradients thus prepared were centrifuged at 4° C in a Hitachi 65P ultracentrifuge using a Hitachi RPS 27.3 swing rotor for 2 h at 27,000 rpm. After centrifugation, the fractions were collected from the top of the gradient with the aid of a Hitachi density fractionator, and radioactivity was counted with a Beckman model LS 250 scintillation spectrometer after scintillator (ACS II, Amersham) had been added.

Detection of DNA cleavage on agarose gel electrophoresis. Strand scission of phage PM2 DNA was detected with the following procedure using the method described by Suzuki et al. [12]. Briefly, 18 μl of the reaction mixture contained 0.5 μg phage PM2 DNA, 50 m*M* Tris-HCl (pH7.6), the desired concentration of KT6149 or MMC, and a reducing agent [1 m*M* dithiothreitol (DTT) or 5 μM $FeSO_4$) where mentioned, and incubation was done at 37° C for 1 h. After the addition of 2 μl 0.1% bromophenol blue in 50% glycerol, the reaction mixture was assessed by 0.8% agarose gel electrophoresis in 25 m*M* Tris-acetate buffer (pH 8.0) at room temperature. After staining with 1 μg/ml ethidium bromide for 30 min, DNA bands were photographed over UV light with a Polaroid type 667 camera.

Alkaline agarose gel electrophoresis of linearized plasmid DNA. To assess the action of KT6149 on linearized plasmid DNA, EcoRI-digested pBR322 was used. For the reaction mixture, 0.5 μg of the linearized pBR322 was dissolved in 50 m*M* Tris-HCl buffer (pH 7.6) in the presence of 1 m*M* DTT and 5 μM $FeSO_4$ with KT6149 (10^{-5} *M*) or MMC (10^{-4} *M*) and incubated at 37° C for 1 h. After precipitation with ethanol, DNA was dissolved in 20 μg loading buffer containing 50 m*M* NaOH, 1 m*M* Na_2-EDTA, 2.5% Ficoll 400, and 0.1% bromophenol blue, then run on 0.9% agarose gels in 30 m*M* NaOH and 1 m*M* Na_2-EDTA. DNA bands were visualized with ethidium bromide as described above.

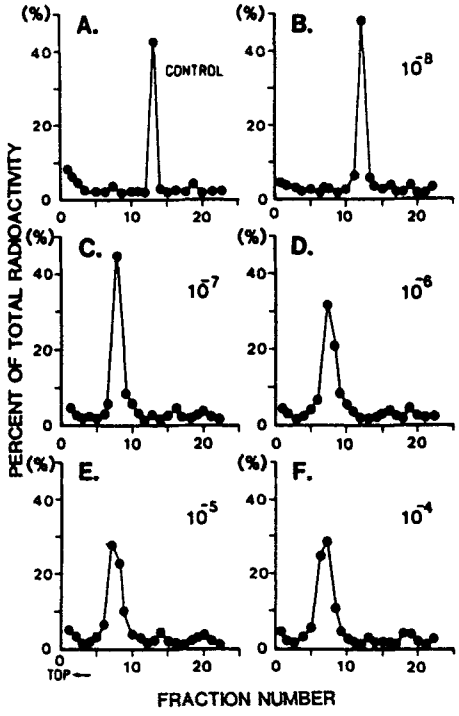

Fig. 4 A–F. Alkaline sucrose density-gradient sedimentation profiles for DNA of HL-60 cells treated with KT6149 for 1 h. The percentage of total radioactivity in each fraction was calculated. The linear gradient ranged from 5% to 20%. KT6149 concentrations used included A 0 (untreated control), B 10^{-8} *M*, C 10^{-7} *M*, D 10^{-6} *M*, E 10^{-5} *M*, and F 10^{-4} *M*

Results

Inhibitory effects of KT6149 on the growth of HL-60 cells

Figure 2 illustrates growth reduction values for HL-60 cells treated with KT6149 or MMC at day 3. The IC_{50} of KT6149 and MMC were 2×10^{-9} *M* and 2×10^{-8} *M*, respectively, the inhibitory effect of KT6149 on the growth of HL-60 cells being 10-fold more potent than that of MMC.

Effects of KT6149 on macromolecular synthesis

Figure 3 illustrates the inhibitory effect of KT6149 on the incorporation of [^{3}H]-thymidine, [^{3}H]-uridine, or [^{3}H]-leucine into the acid-insoluble fraction of HL-60 cells. The incorporation of [^{3}H]-thymidine was most severely inhibited, KT6149 seeming preferentially to block DNA synthesis rather than RNA or protein synthesis at low concentrations. In the MMC case, significant inhibition was observed for [^{3}H]-thymidine incorporation at concentrations higher than 10^{-6} *M*, with almost no effects being observed on the incorporation of [^{3}H]-uridine or [^{3}H]-leucine. The IC_{50} values of KT6149 and MMC for [^{3}H]-thymidine incorporation were 2×10^{-7} *M* and 2×10^{-6} *M*, respectively.

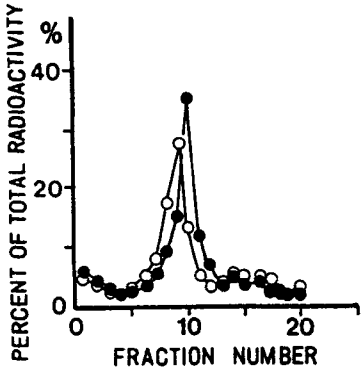

Fig. 5. Alkaline sucrose density-gradient sedimentation profile for DNA of HL-60 cells treated with MMC for 1 h. The linear gradient ranged from 5% to 20%. O——O, untreated control; ●——●, MMC (10^{-5} *M*)

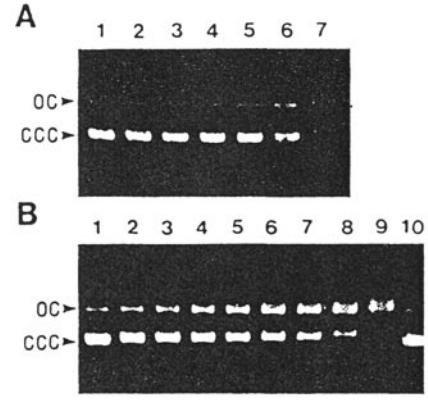

Fig. 6 A, B. Electrophoretic analysis of the interaction of KT6149 with PM2 DNA. A KT6149-induced PM2 DNA cleavage in the presence of DTT. Each reaction was carried out at 37° C for 1 h. 1, drug-free control; 2, 1 mM DTT; 3, 10^{-4} *M* KT6149; 4, 10^{-7} *M* KT6149+1 mM DTT; 5, 10^{-6} *M* KT6149+1 mM DTT; 6, 10^{-5} *M* KT6149+1 mM DTT; 7, 10^{-4} *M* KT6149+1 mM DTT. B Function of the duration of KT6149 drug exposure for PM2 DNA cleavage. Each reaction was carried out at 37° C in the presence of 10^{-5} *M* KT6149 and 1 mM DTT. 1, 0 min; 2, 10 min; 3, 20 min; 4, 30 min; 5, 40 mn; 6, 50 min; 7, 60 min; 8, 120 min; 9, 180 min; 10, 180 min (without DTT)

Alkaline sucrose density-gradient sedimentation profiles for DNA of HL-60 cells treated with KT6149 or MMC

Figure 4 shows the alkaline sucrose density-gradient sedimentation profile for DNA of HL-60 cells treated with KT6149 for 1 h at 37° C. In the controls, the peak of radioactivity was located in fraction 13. However, in the cells treated with KT6149, the peak shifted toward the top of the gradient, the location of the peaks in cells treated with 10^{-8} *M*, 10^{-7} *M*, 10^{-6} *M*, 10^{-5} *M*, and 10^{-4} *M* KT6149 being in fractions 12, 8, 7, 7, and 6, respectively. The results show that single-strand scission of DNA was induced in HL-60 cells by KT6149 in 1 h. With MMC, no significant change in the sedimentation velocity of DNA from HL-60 cells was observed (Fig. 5).

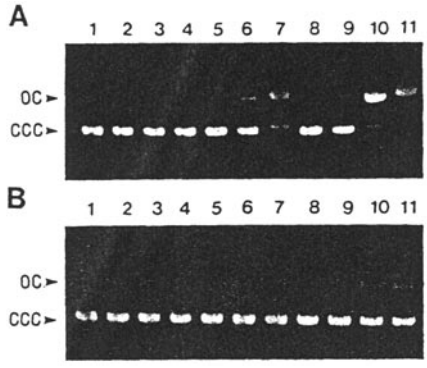

Fig. 7 A, B. Effect of reducing agents and ferrous ions on the interaction of KT6149 with PM2 DNA. A 1, untreated control; 2, 1 mM DTT; 3, 5 μ*M* $FeSO_4$; *4*, 10^{-5} *M* KT6149; *5*, 10^{-4} *M* KT6149; *6*, 10^{-5} *M* KT6149+1 m*M* DTT; *7*, 10^{-4} *M* KT6149+1 mM DTT; *8*, 10^{-5} *M* KT6149+5 μ*M* $FeSO_4$; *9*, 10^{-4} *M* KT6149+5 μ*M* $FeSO_4$; *10*, 10^{-5} *M* KT6149+1 mM DTT+5 μ*M* $FeSO_4$; *11*, 10^{-4} *M* KT6149+1 mM DTT+5 μ*M* $FeSO_4$. **B** Same as above, except that MMC was used in the place of KT6149

Fig. 8. Alkaline agarose gel electrophoresis of linearized pBR322 DNA treated with KT6149 in the presence of 1 m*M* DTT and 5 μ*M* $FeSO_4$. 1, untreated control; 2, 10^{-4} *M* MMC; 3, 10^{-5} *M* KT6149

Electrophoretic analysis of PM2 DNA cleavage induced by KT6149

To determine whether or not KT6149 directly induces DNA strand breaks in vitro, electrophoretic analysis of PM2 DNA was carried out. As illustrated in Fig. 6, PM2 DNA was separated into two bands on agarose gel electrophoresis. According to Adij and Borst [1], the faster-moving band corresponds to the native, covalently closed circular DNA (cccDNA) form; the slower-moving one, to the open circular DNA (ocDNA); and the intermediate band (not visible in the present experiment), to linear DNA. KT6149 alone did not alter the electrophoretic pattern of PM2 DNA, even at a concentration as high as 10^{-4} *M* (Fig. 6A, lane 3). However, in the presence of 1 m*M* DTT as a reducing agent, the electrophoretic patterns of PM2 DNA showed a decrease in cccDNA and an increase in ocDNA at concentrations of KT6149 higher than 10^{-6} *M* (Fig. 6A, lanes 4–7).

These results indicate that KT6149 caused single-strand scission of PM2 DNA in a dose-dependent manner in the presence of 1 m*M* DTT. Figure 6B shows the electrophoretic patterns of PM2 DNA treated with 10^{-5} *M* KT6149 for 0–180 min, the degree of DNA single-strand scission caused by KT6149 in the presence of 1 m*M* DTT clearly depending on the duration of incubation with the drug.

Requirement of reducing agent and ferrous iron for KT6149-induced DNA cleavage

As described above, KT6149 caused single-strand scission of DNA in the presence of 1 m*M* DTT. The effects of other reducing agents were therefore tested. The KT6149-induced DNA breakage was not observed with 10 m*M* 2-mercaptoethanol (data not shown) or with 5 μM $FeSO_4$ (Fig. 7, lanes 8, 9). However, by combining $FeSO_4$ (5 μM) and DTT (1 m*M*), the degree of KT6149-induced DNA cleavage was markedly elevated (Fig. 7, lanes 10, 11).

Alkaline agarose gel electrophoresis of linearized pBR322 DNA treated with KT6149

To determine the direct action of KT6149 on DNA cross-linking in vitro, linearized pBR322 DNA was selected in this study. Figure 8 illustrates the results of alkaline agarose gel electrophoresis of linearized pBR322 DNA treated with KT6149 or MMC in the presence of 1 m*M* DTT and 5 μM $FeSO_4$. The electrophoretic mobility of KT6149-treated pBR322 DNA was markedly decreased (lane 3), whereas mobility in the MMC-treated plasmid did not show any significant change in comparison with that of control (see lanes 1, 2). This suggests that KT6149 caused interstrand DNA cross-linking.

Discussion

Mitomycin C (MMC), one of the potent antineoplastic agents produced by *Streptomyces caespitosus* [6], contains several biologically active groups, including an aziridine ring, a carbamoyl moiety, and a quinone ring. MMC, which is known to be a bifunctional alkylating agent, mainly forms interstrand DNA and DNA-protein cross-linking [10], alkylation taking place preferentially at the N-2 position of guanine [13].

Although a number of MMC derivatives have been synthesized and tested in attempts to enhance antitumor activity and diminish toxicity, to date the parent compound remains both experimentally and clinically the most potent drug.

However, KT6149 is a novel MMC derivative whose development may enable superior treatment of malignancies, the ultimate hope being its clinical availability. This new compound possesses a side chain, which is linked to the nitrogen atom N-7 of MMC (Fig. 1), and the present examination of its effects on HL-60 cells in vitro demonstrated a cytotoxic potential for the drug 10 times higher than that of MMC (Fig. 2). Furthermore, DNA synthesis was preferentially inhibited with this compound, with comparatively weak inhibition of RNA and protein synthesis being observed. Moreover, the inhibition of DNA synthesis associated with KT6149 application was 10 times that of MMC (Fig. 3).

Using alkaline elution analysis, Akinaga and Morimoto [2] showed that KT6149 formed interstrand DNA and DNA-protein cross-links after 1 h exposure of HeLa S_3 cells to the drug. They reported the cross-linking activities of the drug to be 20 times those of MMC. These authors also demonstrated that strand breaks were not a feature in cells treated with KT6149. Our data also indicated that the drug caused DNA cross-linking, with treatment for 1 h giving rise to a decrease in the electrophoretic mobility of linearized pBR322 DNA in alkaline agarose gels in the presence of DTT (Fig. 8). However, a significant decrease in the sedimentation velocity of DNA of KT6249-treated HL-60 cells in alkaline sucrose gradients was observed (Fig. 4), and single-strand scission was also noted in isolated phage PM2 DNA exposed to the drug (Fig. 6). These results, taken together, strongly suggest that DNA is one of the main targets of KT6149 and that the lesions induced by the drug lead to inhibition of RNA and protein synthesis in the cells, as with MMC.

KT6149 induced both cross-linking of DNA and strand breaks of isolated DNA in a cell-free system. However, only single-strand breaks were detectable in HL-60 cells in the present study. With MMC, single-strand breaks have been observed for supercoiled phage PM2 DNA [7], and the production of chromosomal breaks has been reported [4, 9].

It has been considered that breakage of DNA is attributable to free-radical intermediates (especially the generation of reactive oxygen species) and, partly, to lack of repair of alkylation damage. Therefore, the DNA single-strand scission caused by KT6149 as well as MMC might give rise to cytocidal effects. However, it is not clear why KT6149 induced single-strand DNA scission in HL-60 cells, whereas DNA from HeLa S_3 cells showed cross-linking. Possible explanations for this discrepancy might involve differences in the nature of the individual cells or in the assay methods adopted; however, the discrepancy remains open for discussion.

In general, MMC is activated by chemical (i.e., sodium borohydride) or enzymatic (i.e., NADPH-dependent reductase system) reduction, and its reduced form is responsible for alkylation of DNA and induces single-strand scission. In the present study, KT6149 administration resulted in DNA lesions in the presence of DTT, whereas MMC did not cause any changes (Fig. 6). This finding indicates the possible differences in the mode of activation of the two drugs. Furthermore, cleaving activity for DNA was enhanced by the addition of ferrous ion (Fig. 7). The requirement of a reducing agent and ferrous ion for KT6149-induced DNA strand scission suggests that the production of reactive oxygen species contributes to the DNA cleavage, as is reported to be the case for bleomycin and other anthracyclines [8, 11]. More precise clarification of the mechanisms of action of the drug awaits further studies.

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