Jpn. J. Cancer Res. 85, 418–425, April 1994

A Novel Antitumor Antibiotic, KW-2189 Is Activated by Carboxyl Esterase and Induces DNA Strand Breaks in Human Small Cell Lung Cancer Cells

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KW-2189 has been selected as a lead compound for clinical trial among duocarmycin derivatives with structural similarity to CC-1065, a cyclopropylpyrroloindole. The purpose of this study was to examine the DNA-binding potency and the mechanisms of cytotoxicity of KW-2189. In order to analyze DNA-binding activity of KW-2189, plasmid pBR322 was treated with KW-2189 with or without pretreatment with carboxyl esterase, which we demonstrated to be an activating enzyme, and the products were examined by agarose gel electrophoresis and restriction enzyme analysis. Cytotoxic activity was examined by exposing a human small cell lung cancer cell line, NCI-H69 to KW-2189 with or without carboxyl esterase. Alkaline elution was performed to examine whether KW-2189 induces DNA strand breaks. DNA treated with KW-2189 and carboxyl esterase migrated faster than KW-2189-treated DNA, which migrated at the same rate as untreated DNA. In addition DNA treated with esterase-activated KW-2189 was protected from digestion by some restriction enzymes. KW-2189 showed concentration- and time-dependent growth inhibitory effect with IC_{50} values (drug concentration required for 50% growth inhibition) of 58 n*M* (96 h) to 1900 n*M* (1 h) in H69 cells. The IC_{50} values of 4-h exposure of H69 to KW-2189 with 0, 26, 130, 650 mU/ml carboxyl esterase were 460, 120, 30, and 7 n*M*, respectively. Time-dependent enhancement of cytotoxicity by carboxyl esterase was also observed. KW-2189 induced DNA strand breaks in H69 cells in a concentration-dependent manner around the IC_{50} value. We conclude that 1) KW-2189 is activated by carboxyl esterase to its active form(s), 2) activated KW-2189 has a stronger DNA-binding activity and cytotoxicity than KW-2189, 3) DNA cleavage is one of the major mechanisms of KW-2189-mediated cytotoxicity.

Key words: KW-2189 — Cytotoxicity — DNA strand break — Carboxyl esterase — Drug activation

Investigation of the mechanisms of cytotoxicity is important not only to understand the biological potency of the drug of interest but also to facilitate synthesizing effective anticancer drugs. Recent advances have led to the development of more effective and less toxic drugs such as topoisomerase inhibitors and tubulin-interacting agents.[1-8] In comparison to adriamycin, CC-1065 was 100-fold more potent against a broad panel of human tumors in a cloning assay.[9] In spite of its superior anticancer activity, CC-1065 was not introduced into clinical trial because of its delayed lethal toxicity to mice.[10] Many anticancer drugs that have similar structure to CC-1065 have been reported.[11-14] Adozelesin, a CC-1065 derivative that does not show delayed lethal toxicity, is under clinical trial in the United States. Duocarmycins (DUMs) were isolated from *Streptomyces* sp. at Kyowa Hakko Kogyo Co., Ltd., Tokyo, as novel anticancer drugs with structural similarity to CC-1065.[15] DUMs are composed of duocarmycins A, B1, B2, C1, and C2. They are active against a broad spectrum of tumors such as nasopharyngeal carcinoma, LB cells and breast cancer, MCF-7 cells and adriamycin-resistant sublines.[16] Poor water solubility is a problem with DUMs, so various duocarmycin derivatives with improved solubility and cytotoxicity have been synthesized at Kyowa Hakko. KW-2189 (methyl (1S)-1-bromomethyl-7-methyl-5-[(4-methylpiperazinyl)-carbamyloxy]-3-[(5,6,7-trimethoxy-indol-2-yl)-carbonyl]-1,2-dihydro-3*H*-pyrrolo[3,4-*e*]indole-8-carboxylate hydrobromide), which was synthesized as a water-soluble derivative of duocarmycin B2, has the structure shown in Fig. 1. It was active against cisplatin-resistant human non-small cell lung cancer cell lines and a multidrug-resistant human small cell lung cancer cell line *in vitro*.[17] Because of its superior anticancer activity and lower toxicity, KW-2189 was selected for clinical trial in Japan.[18] The purpose of this study was to examine the DNA-binding potency and to determine the mechanisms of cytotoxicity of KW-2189.

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[4] Awardee of a Research Resident Fellowship from the Foundation for Promotion of Cancer Research.

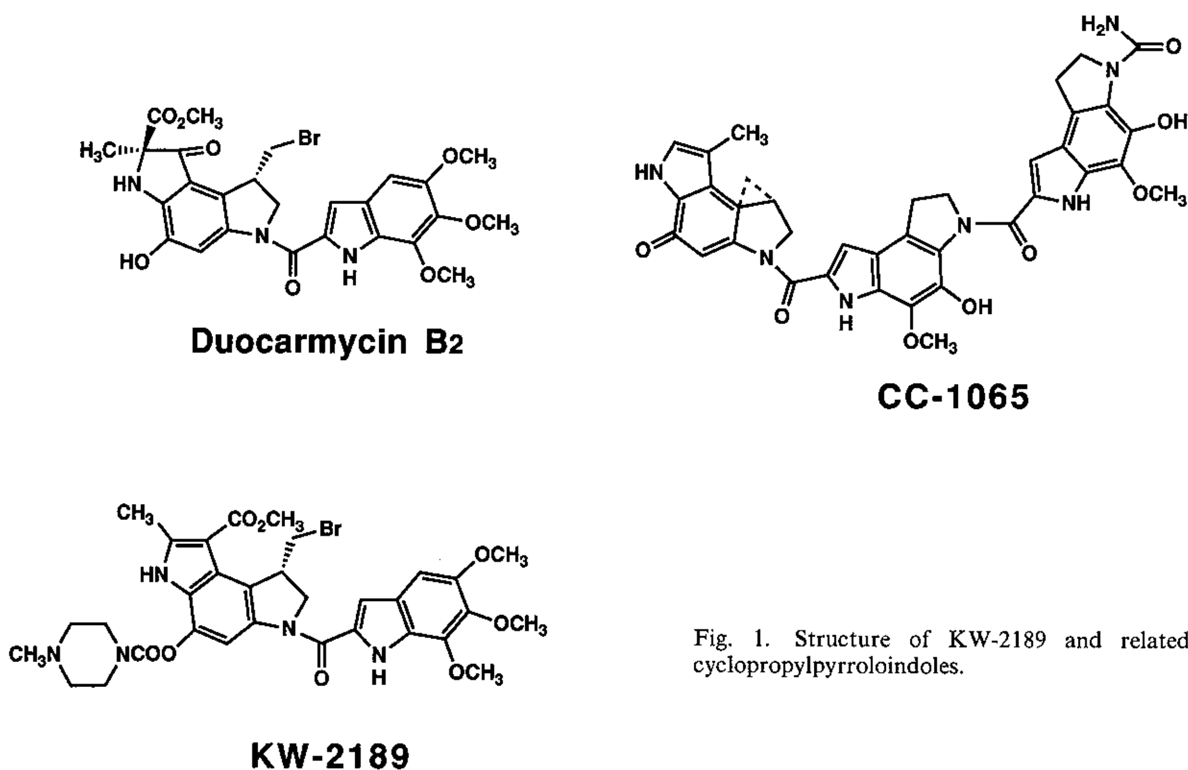

Fig. 1. Structure of KW-2189 and related cyclopropylpyrroloindoles.

MATERIALS AND METHODS

Chemicals KW-2189 was supplied by Kyowa Hakko Kogyo Co., Tokyo. The compound was dissolved in 5% glucose solution (Otsuka Pharmacy Co., Tokyo) and stored at −80°C. Carboxylic-ester hydrolase (carboxyl esterase) was obtained from Boehringer Mannheim Yamanouchi, Tokyo, and diluted in double-distilled water just before use. RPMI 1640 and phosphate-buffered saline without metal salts (PBS(−)) were purchased from Nissui, Tokyo. [methyl-^{14}C]Thymidine (52 mCi/mmol) was obtained from Amersham, Buckinghamshire, England, proteinase K from E. Merck AG, Darmstadt, Germany, tetrapropylammonium hydroxide from Eastman Kodak Co., Rochester, NY. Restriction enzymes were obtained from Toyobo Co., Osaka. Other chemicals were purchased from Sigma Chemical Co., St. Louis, MO, unless otherwise stated.

Measurement of DNA-binding activity If the KW-2189 molecule binds to DNA, DNA will be protected from intercalation of ethidium bromide. A difference in the amount of ethidium bromide intercalating into DNA results in a different DNA mobility upon agarose gel electrophoresis.[19] A 20-μl reaction mixture containing 500 ng of pBR322 DNA, which had been stored (250 ng/μl) in TE (10 m*M* Tris-HCl, pH 7.9, 1.0 m*M* EDTA) at −20°C, and KW-2189 and/or carboxyl esterase was incubated at 37°C for 4 h in the dark. The resultant samples were subjected to electrophoresis on 1.2% agarose gel in TAE buffer (0.04 *M* Tris-acetate and 0.001 *M* EDTA, pH 8.0) containing 0.5 μg/ml ethidium bromide. Electrophoresis was performed at 2 V/cm for 15 h.

Restriction enzyme analysis KW-2189 (10 μM) was incubated with carboxyl esterase at 37°C for 4 h, then the mixture was heated to 60°C for 30 min to inactivate the enzyme. pBR322 DNA was treated with esterase-treated KW-2189 at 37°C for 2 h. The resultant sample was extracted with phenol followed by ethanol precipitation. The reaction mixture, containing 10 U of restriction enzyme and drug-treated DNA, was incubated at 37°C for 2 h.

Cell cultures NCI-H69 is a human small cell lung cancer cell line. Cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Cytosystem, Australia) plus penicillin (100 U/ml) and streptomycin (100 μg/ml) in a humidified 5% CO_2 atmosphere at 37°C.

Cytotoxic activity of KW-2189 and effect of carboxyl esterase MTT assay was performed as reported by Mosmann.[20] For the continuous exposure experiments, cell suspension (2×10^4/ml) was seeded in 96-well microplates and incubated with KW-2189 for 96 h. For the

short-term exposure, after 2-h incubation of 2.9 ml of cell suspension (2×10^4/ml), 0.1 ml of KW-2189 solution was added. After the drug exposure, cells were washed twice with warmed fresh culture medium. Cells were adjusted to 2×10^4/ml again in drug-free medium, and 200 μl aliquots of cell suspension were seeded in 96-well microplates, and incubated until 96 h after the start of drug exposure. The effect of carboxyl esterase on KW-2189-induced cytotoxicity was also examined. Cells were treated with KW-2189 for 4 h and 130 mU/ml carboxyl esterase was added at various times to activate KW-2189 for 1, 2, and 4 h before washing of the cells. To examine the activity-dependent drug-activation by carboxyl esterase, various amounts of carboxyl esterase were added simultaneously with KW-2189 for 4 h.

Alkaline elution assay Alkaline elution assay was performed as described by Kohn *et al.*[21] Cells were prelabeled with [methyl-^{14}C]thymidine (0.075 mCi/ml) for 60 h. The ^{14}C-labeled cells (5×10^5/dish) were treated with 200 n*M* KW-2189 with or without 130 mU/ml carboxyl esterase for 1, 2, 3, 4, or 6 h to examine the time-depednent DNA strand breakage. To examine the concentration-dependent DNA strand breakage, cells were treated with various concentrations of KW-2189 for 4 h. Drug-treated cells were then diluted in cold PBS($-$), gently deposited onto a polycarbonate filter (2.0 μm pore size, 25 mm diameter, Costar Corp., Cambridge, MA) and rinsed with cold PBS($-$). The cells were lysed with 5 ml of lysis solution containing 2% sodium dodecyl sulfate, 25 m*M* disodium EDTA, 50 m*M* Tris, 50 m*M* glycine, and proteinase K (0.5 mg/ml), pH 10.0, for 1 h. This lysis solution was allowed to flow through the filter under gravity, and then the filter was rinsed 3 times with 3 ml of 20 m*M* disodium EDTA, pH 10.0. DNA was eluted with 30 ml of tetrapropylammonium hydroxide-tetrahydroxy-EDTA, pH 12.1 at a constant flow rate of 0.03 ml/min. To analyze dose-dependent DNA strand breaks, ten fractions of the eluate were collected directly into scintillation vials at 1.5-h intervals for 15 h. One-milliliter aliquots of total eluate were used to analyze time-dependent DNA strand breaks. Rad equivalents were calculated as described by Kohn *et al.*[21]

RESULTS

Effect of carboxyl esterase on the DNA-binding activity of KW-2189 Plasmid DNA treated with only KW-2189 or carboxyl esterase migrated at the same rate as untreated DNA on the agarose gel. When DNA was treated with KW-2189 and carboxyl esterase together, open circular DNA migrated faster, and the mobility depended on both the concentration of KW-2189 and the activity of carboxyl esterase (Fig. 2). These data suggested that the altered mobility of DNA was due to the formation of drug-DNA adducts and that carboxyl esterase facilitated the DNA-binding activity of KW-2189. The different mobility of open circular DNA was thought to reflect the amount of adduct formation. There was no mobility shift in closed circular DNA.

Restriction enzyme analysis Plasmid DNA exposed to KW-2189 or esterase-treated KW-2189 was incubated with restriction enzymes, *Eco*R V, *Dra* I, and *Hin*d III. Drug-adducted DNA was expected to be protected from cleavage by restriction enzyme, if there are drug-DNA adducts in the sequence recognized by the restriction enzyme. Plasmid pBR322 contains one *Eco*R V site yielding linearized 4363 base pair (bp) fragments, three *Dra* I sites yielding 3651, 692, and 20 bp fragments, and one *Hin*d III site yielding linearized fragments. When pBR322 (500 ng) treated only with 10 μM KW-2189 for 4 h was incubated with *Eco*R V, very little closed circular DNA remained (Fig. 3a, lane 5). On the other hand, pBR322 treated with 10 μM KW-2189 preincubated with 1300 mU/ml carboxyl esterase was not digested completely by *Eco*R V (Fig. 3a, lanes 7, 9, 11 and 13). When DNA was treated with esterase-treated KW-2189 for 30 min, some DNA remained in the closed circular form. The amount of closed circular DNA increased time-dependently until 1 or 2 h. While a small amount of the open circular DNA remained when DNA was treated

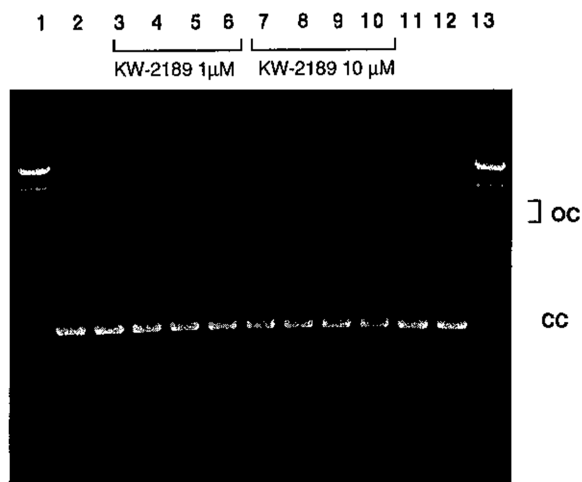

Fig. 2. DNA agarose gel electrophoresis. Plasmid pBR322 DNA (500 ng) was incubated with KW-2189 and/or carboxyl esterase. Electrophoresis was performed at 2 V/cm for 15 h. Lanes 1 and 13 are λ/*Hin*d III DNA molecular markers. Lanes 2 and 12 are untreated pBR322. Concentration of KW-2189 was 1 μM (lanes 3–6), 10 μM (lanes 7–10), or 0 (lane 11). Activity of carboxyl esterase was 0 (lanes 3, 7), 13 mU/ml (lanes 4, 8), 130 mU/ml (lanes 5, 9) or 1300 mU/ml (lanes 6, 10 and 11). OC and CC indicate the positions of open circular and closed circular DNA, respectively.

only with KW-2189, pretreatment of KW-2189 with carboxyl esterase resulted in a time-dependent increase of the amount of remaining open circular DNA. This result suggested that KW-2189 itself had little DNA-binding activity and that carboxyl esterase enhanced the DNA-binding activity of KW-2189. Increased amounts of open circular DNA suggest that *Eco*R V digested only the opposite strand to the drug-adducted site. We also conducted similar analyses using *Dra* I and *Hin*d III (Fig. 3b). Plasmid DNA (250 ng) was incubated with KW-2189 or esterase-treated KW-2189 for 2 h. When pBR322 was treated with only KW-2189 and digested by *Dra* I, DNA was digested as completely as untreated DNA. On the contrary, plasmid DNA treated with esterase-treated KW-2189 was not digested completely by *Dra* I. When DNA was treated with esterase-treated KW-2189 and digested by *Dra* I, 3651-bp fragments and 692-bp fragments disappeared, and new ones appeared (Fig. 3b, lane 8). There were open circular fragments (upper band) and fragments of about 4300 bp (lower band). When untreated DNA or KW-2189-treated DNA was digested by *Hin*d III, linearized fragments appeared. On the other hand, DNA treated with esterase-treated KW-2189 afforded no linearized fragments, but only closed circular and open circular DNA (Fig. 3b, lane 11). These data suggested that KW-2189 itself could not bind at the sequences recognized by *Dra* I and *Hin*d III and that carboxyl esterase facilitated DNA-binding of KW-2189 at least around the sequences recognized by these restriction enzymes.

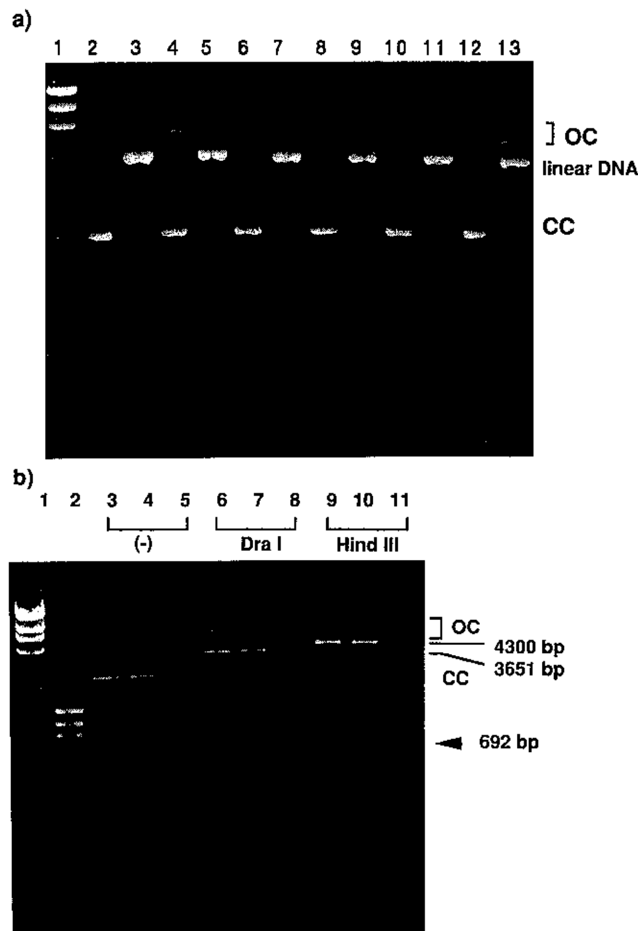

Fig. 3. Restriction enzyme analysis. KW-2189 (10 μM) was incubated with 1300 mU/ml carboxyl esterase for 4 h, then the mixture was heat-treated at 60°C for 30 min to inactivate the enzyme. (a) Plasmid pBR322 (500 ng) was treated with KW-2189 for 4 h (lanes 4 and 5) or esterase-treated KW-2189 for 30 (lanes 6 and 7), 60 (lanes 8 and 9), 120 (lanes 10 and 11), or 240 min (lanes 12 and 13). Lane 1 is molecular markers (λ/*Hin*d III). Lane 2 is untreated DNA and lane 3 is untreated DNA digested by *Eco*R V. The samples in lanes 4, 6, 8, 10, and 12 are drug-treated DNA and those in lanes 5, 7, 9, 11 and 13 are drug-treated DNA digested by restriction enzyme *Eco*R V. (b) pBR322 (250 ng) was treated with KW-2189 or esterase-treated KW-2189 for 2 h. Lanes 1 and 2 are molecular markers (lane 1; λ/*Hin*d III, lane 2; $\phi\chi$174/*Hae* III). Lanes 3, 6 and 9 are untreated DNA, lanes 4, 7 and 10 are DNA treated with KW-2189, and lanes 5, 8 and 11 are DNA treated with esterase-treated KW-2189. The samples in lanes 6–8 and samples in lane 9–11 were digested by *Dra* I or *Hin*d III, respectively. OC, open circular DNA; CC, closed circular DNA.

Cytotoxicity of KW-2189 and effect of carboxyl esterase
The IC_{50} values of the 1, 2, 4, and 96-h KW-2189 exposures of H69 were 1900, 1020, 460, and 58 nM as

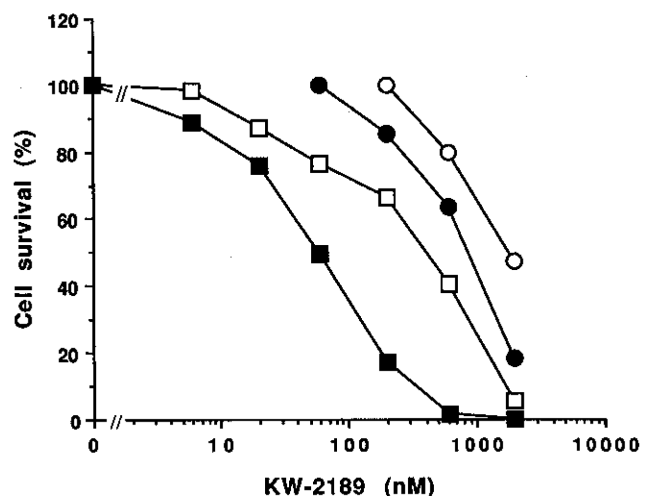

Fig. 4. Cytotoxic activity of KW-2189. Human small cell lung cancer cells NCI-H69 were treated with KW-2189 for 1 (○), 2 (●), 4 (□), and 96 h (■). Cell survival was measured by MTT assay 96 h after the start of drug treatment. The cells treated for 96 h were not washed. Values are the average of three experiments.

shown in Fig. 4. The results of the restriction enzyme analysis suggested that KW-2189 itself has only a little DNA binding activity and that carboxyl esterase increased the DNA-binding activity of KW-2189. Based on this idea, we examined whether carboxyl esterase could enhance the cytotoxicity of KW-2189. While 1300 mU/ml carboxyl esterase did not inhibit the growth of H69 (data not shown), the cytotoxicity of a 4-h exposure of KW-2189 was enhanced time-dependently by 130 mU/ml carboxyl esterase (Fig. 5). When cells were treated simultaneously for 4 h with both KW-2189 and 26, 130, or 650 mU/ml of carboxyl esterase, the IC_{50} values of KW-2189 decreased to 120, 30, and 7 n*M*, respectively (Fig. 6).

DNA strand breaks Using the alkaline elution technique, we examined whether KW-2189 could cleave cellular DNA. Whether carboxyl esterase can enhance the effect of KW-2189 on the cellular DNA was also examined. When cells were treated with KW-2189 for 2 h, no DNA strand breaks were detected even at 2000 n*M* KW-2189 in the preliminary experiment. This result suggested that KW-2189 could not cleave cellular DNA or that it cleaved DNA very slowly. We then examined time-dependent DNA cleavage by KW-2189. Two hundred nanomolar KW-2189 showed 53, 80, and 106 rad equivalent DNA strand breaks after 3-, 4-, and 6-h treatment, respectively (Fig. 7). This concentration was much lower than the IC_{50} value (460 n*M*) of 4-h exposure of KW-2189. Viability of the cells examined by trypan blue staining was more than 90% cells after treatment with 2000 n*M* KW-2189 for 6 h (data not shown). When cells were treated with both KW-2189 and 130 mU/ml

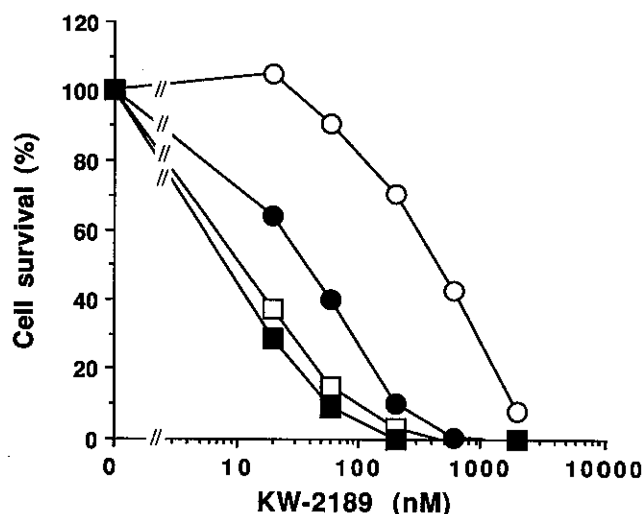

Fig. 6. Activity-dependency of the effect of carboxyl esterase on the cytotoxicity of KW-2189. Cells were exposed to various concentrations of KW-2189 and carboxyl esterase for 4 h followed by 92-h incubation in drug-free medium. The activity of carboxyl esterase was fixed at 0 (○), 26 mU/ml (●), 130 mU/ml (□), 650 mU/ml (■). Cell survival was determined by MTT assay. Values are the average of three experiments.

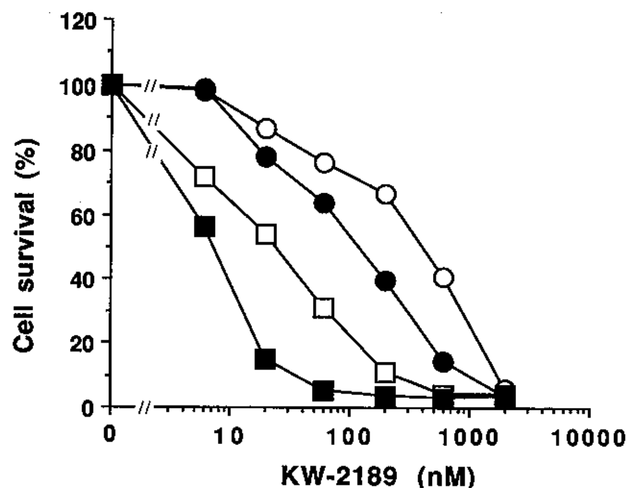

Fig. 5. Time-course of the effect of carboxyl esterase on the cytotoxicity of KW-2189. Cells were exposed to various concentrations of KW-2189 for 4 h with or without (○) carboxyl esterase followed by 96-h incubation in drug-free medium. Carboxyl esterase (130 mU/ml) was added during KW-2189 treatment 1 h (●), 2 h (□), or 4 h (■) before cell washing. Cell survival was determined by MTT assay. Values are the average of three experiments.

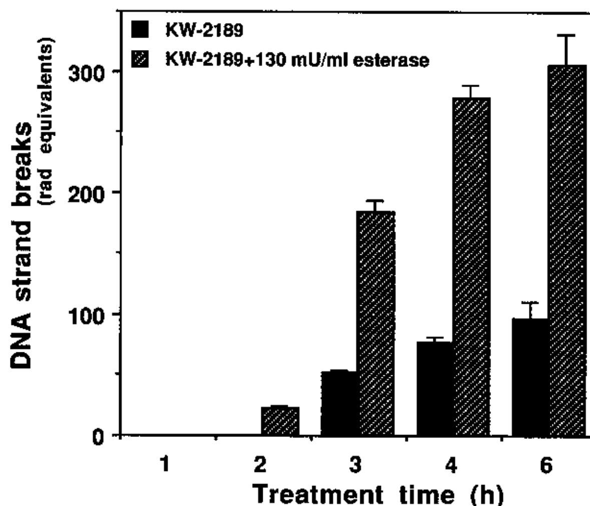

Fig. 7. Time-dependent induction of DNA strand breaks by KW-2189. Cells were treated with 200 n*M* KW-2189 with or without 130 mU/ml carboxyl esterase for 1, 2, 3, 4, and 6 h. DNA strand breaks were examined by the alkaline elution assay just after treatment. Values are means of three experiments ± SD. DNA strand-breakage frequencies are expressed as rad equivalents.

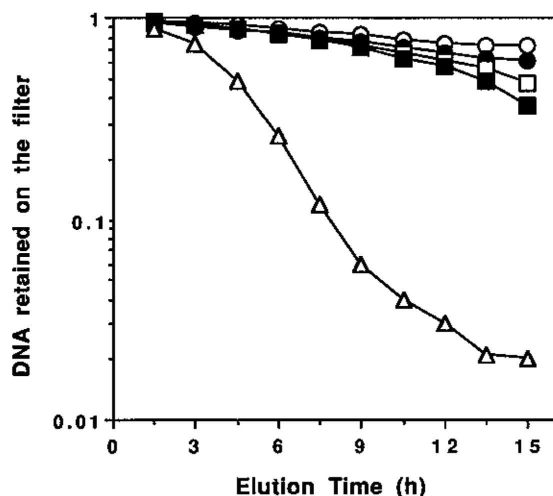

Fig. 8. Dose-dependent induction of DNA strand breaks by KW-2189. Cells were exposed to 0 (○); 20 n*M* (●); 200 n*M* (□); 2000 n*M* (■) KW-2189 or 20 n*M* KW-2189+130 mU/ml carboxyl esterase (△) for 4 h. Values were measured as labeled DNA retained on the filter. Each symbol represents the mean of two experiments.

carboxyl esterase, DNA strand breaks were detected after 2 h and there were about 300 rad equivalent of DNA strand breaks (over 90% of labeled DNA was eluted) after 4 h. This result suggested that KW-2189-mediated DNA-cleavage was time-dependent and could be augmented by carboxyl esterase. When cells were treated for 4 h with 20, 200, 2000 n*M* KW-2189, DNA strand breakage occurred in a concentration-dependent manner (Fig. 8). When cells were treated with 20 n*M* KW-2189 and 130 mU/ml carboxyl esterase, most of the DNA (approximately 98%) was eluted.

DISCUSSION

Our first experiment on KW-2189 *in vitro* had been designed to examine the concentration-dependent DNA mobility shift upon agarose gel electrophoresis. In spite of the potent cytotoxic activity of KW-2189, this preliminary experiment suggested that KW-2189 could not bind with DNA. We hypothesized that KW-2189 was a prodrug metabolized in cells to a form with higher binding ability to DNA. The N-methylpiperazine side chain, which confers water-solubility upon KW-2189, might need to be removed for the activation of KW-2189. In the presence of carboxyl esterase, KW-2189-treated DNA showed a mobility shift upon agarose gel electrophoresis. This mobility shift was observed only in open circular DNA and not in closed circular DNA. Two possibilities were considered: KW-2189 could not bind to closed circular DNA, or KW-2189 could bind to both closed circular and open circular DNA, but the conformational change after adduct formation in closed circular DNA was too small to be detected by this assay. To examine these questions as well as to confirm that mobility shift in this assay reflected the formation of drug-DNA adduct, restriction enzyme analysis was performed. The results demonstrated that KW-2189 formed a drug-DNA adduct in both closed and open circular DNA after incubation with carboxyl esterase. In this assay, carboxyl esterase was inactivated before treatment of DNA. We conclude KW-2189 was activated by this enzyme. Based on these results, KW-2189 is considered to be a prodrug with little DNA-binding activity, and carboxyl esterase metabolizes KW-2189 into its active form(s), probably by removing the N-methylpiperazine side chain.

We demonstrated that the cytotoxicity of KW-2189 was enhanced time- and activity-dependently by carboxyl esterase. These results strongly suggest that carboxyl esterase enhances the cytotoxicity of KW-2189 by facilitating the DNA-binding of KW-2189. Hence, activity of carboxyl esterase in cells may be one of the factors that influence the sensitivity of cancer cells to KW-2189. It would be interesting to examine the relationship between carboxyl esterase activity in cancer cells and the antitumor effect.

CC-1065, which has been most intensively studied among cyclopropylpyrroloindoles (CPIs), alkylates DNA through N_3 of adenine in a highly sequence-specific manner in the minor groove of double-stranded DNA.[22,23] CC-1065 inhibits DNA polymerization and helicase-mediated unwinding of DNA.[24,25] It has also been reported that DNA treated with CC-1065 and its analogs was cleaved after thermal treatment in cell-free systems.[26] The mechanisms of cytotoxicity of CPIs in cellular systems have not been determined yet. One CC-1065-analogue, U-77,779, was reported to form DNA interstrand cross-links when cells were treated with an extremely high drug concentration for 6 h, as measured by the technique of alkaline elution.[12] Adozelesin, selected as a lead compound from among analogues of CC-1065 for clinical trials, was reported not to form any single-strand-breaks, interstrand cross-links, or DNA-protein cross-links.[12] Duocarmycin B1 (DUMB1) was reported to have DNA-cleaving activity for tumor cells at a concentration of 100 times its IC_{50}.[16] Therefore it does not seem that DNA strand breakage is a major mechanism of cytotoxicity of CPIs. We demonstrated that KW-2189 induced DNA strand breaks at concentrations around the IC_{50}. The frequency of DNA strand breakage depended on the concentration of KW-2189 and was enhanced by carboxyl esterase. The results of alkaline elution assay were consistent with the results on cytotoxic

activity of KW-2189. These data suggested that DNA-cleaving activity was at least one of the major mechanisms of cytotoxicity of KW-2189, and that the enhanced cytotoxicity of KW-2189 by carboxyl esterase was due to increased drug-DNA adduct formation, leading to DNA-strand breakage. Thus, KW-2189 exhibits a unique mechanism of cytotoxicity among CPIs. However, the structure of the activated KW-2189 was not determined; small differences in side chain structure may alter the antineoplastic activity and the mechanisms of actions of CPIs.

It is important to know the structures of the metabolites of KW-2189, and what proportion of KW-2189 will be activated by carboxyl esterase. Examination of the relative cytotoxicity of KW-2189 and its activated form(s) is also important. Such information will be of value in clinical trials.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid from the Ministry of Health and Welfare for the Comprehensive 10-Year Strategy for Cancer Control, Japan and by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

(Received October 16, 1993/Accepted January 6, 1994)

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