RAPID COMMUNICATION Jpn. J. Cancer Res. 88, 1033–1037, November 1997

In vitro Enhancement of Antitumor Activity of a Water-soluble Duocarmycin Derivative, KW-2189, by Caffeine-mediated DNA-repair Inhibition in Human Lung Cancer Cells

Hayato Ogasawara, Kazuto Nishio, Tomoyuki Ishida, Hitoshi Arioka, Kazuya Fukuoka and Nagahiro Saijo[1]

Pharmacology Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104

Duocarmycins, including KW-2189, bind in the minor groove of double-stranded DNA at A-T-rich sequences, followed by covalent bonding with N-3 of adenine in preferred sequences. We examined the effect of DNA-repair modulators, such as caffeine and aphidicolin, on the cytotoxicity of duocarmycins towards human lung cancer cells, as determined by dye formation assay. Caffeine (0.5 or 1 m*M*), but not aphidicolin, enhanced the growth-inhibitory activity of KW-2189, DU-86, and duocarmycin SA. Caffeine inhibited repair of DNA strand breaks induced by KW-2189, as assayed by the alkaline elution technique. This suggests that duocarmycin-induced DNA strand breaks, which are potentially lethal to cells, are repaired through a caffeine-sensitive pathway.

Key words: Duocarmycin — DNA strand breaks — Excision repair — KW-2189 — UV-irradiation

KW-2189 is a novel water-soluble derivative of duocarmycin B2, originally isolated from *Streptomyces* subspecies at Kyowa Hakko Kogyo Co., Ltd. (Tokyo). The duocarmycins, which consist of duocarmycins A, B1, B2, C1, C2 and SA, and their derivatives, have similar structures and share a pharmacophore, the cyclopropylpyrroloindole (CPI) moiety, in common with CC-1065, which was isolated from *Streptomyces zelensis* at Upjohn Co. (Kalamazoo, MI) (Fig. 1).[1)] Poor water-solubility and poor stability have made the duocarmycins difficult to use. Thus, various duocarmycin derivatives with superior solubility and stability have been synthesized at Kyowa Hakko Kogyo Co. Among them, methyl(1*S*)-1-bromomethyl-7-methyl-5-[(4-methylpiperazinyl)-carbonyloxy]-3-[5,6,7-trimethoxy-2-indolyl)-carbonyl]-1,2-dihydro-dH-pyrrolo[3,2-*e*]indole-8-carboxylate hydrobromide (KW-2189) was selected for clinical trials. KW-2189 has been shown to be more active than so-called "key agents," such as cyclophosphamide, mitomycin C, adriamycin and cisplatin, against various human cancer cell lines inoculated into mice.[2)] We are interested in the biochemical basis of the growth-inhibitory activity of KW-2189, since it is a promising drug for clinical application. We have reported that carboxyl esterase increased the DNA-binding activity of KW-2189 and that KW-2189 produced cellular DNA strand breaks after a lag period of approximately 3 h, an effect which was also augmented by carboxyl esterase.[3,4)] As suggested by the structural similarity between CC-1065 and duocarmycins, the chemical properties of these two agents have been proven to be similar. Duocarmycins, CC-1065 and their analogs bind through the N-3 position of adenine in the DNA minor groove in a sequence-specific manner to form a CPI-DNA adduct.[5)] Drug-adducted DNA induced by KW-2189 was refractory to digestion by restriction endonucleases[4)] and DNase I (Ogasawara, unpublished data), in agreement with observations on the CC-1065-DNA adduct.

DNA repair is an important process that determines the sensitivity of a cell to ultraviolet irradiation and chemical damage,[5–7)] therefore it is of interest to determine whether the CPI-DNA adduct can be repaired by nucleotide excision repair (NER), the mechanism by which conventional drug-DNA adducts are repaired.[8,9)] In addition, since it is generally recognized that most bulky DNA lesions inhibit DNA replication, it seems valuable to examine whether DNA repair machinery can be inhibited by certain types of DNA lesions. The aim of our current study was to investigate the relationship between the repair ability and the cytotoxicity of duocarmycins. We examined the kinetics of repair of DNA cleavage induced by KW-2189 by the alkaline elution method.

KW-2189, DU-86,[4)] an activated form of KW-2189 and duocarmycin SA were supplied by Kyowa Hakko Kogyo Co. Carboxylic-ester hydrolase (carboxyl esterase) was obtained from Boehringer Mannheim Yamanouchi, Tokyo, and diluted in double-distilled water just before use. [Methyl-^{14}C]thymidine (52 mCi/mmol) was obtained from Amersham, Buckinghamshire, England, proteinase K from E. Merck AG, Darmstadt, Germany, tetrapropylammonium hydroxide from East-

[1] To whom all correspondence should be addressed.

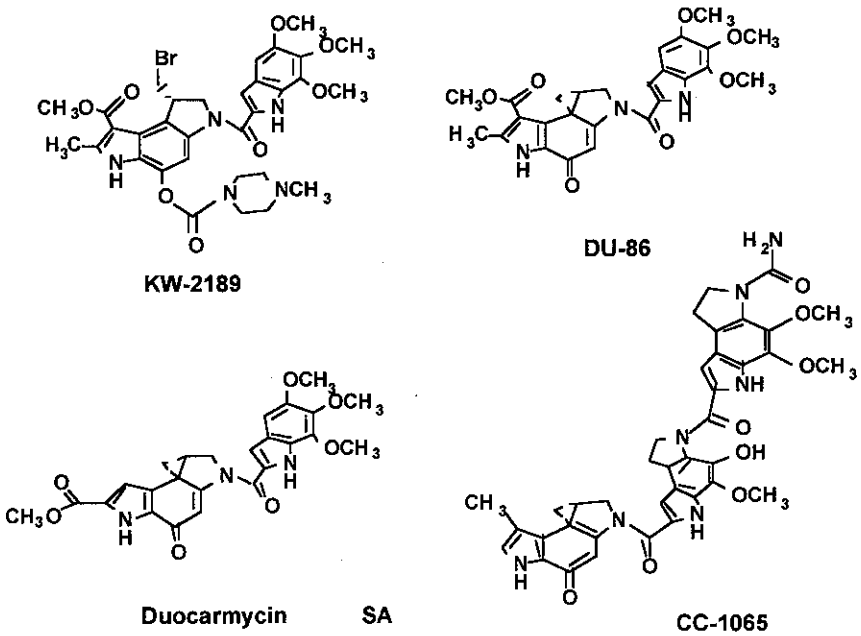

Fig. 1. Structure of KW-2189 and related cyclopropylpyrroloindoles.

man Kodak Co., Rochester, NY. *Eco*R I was obtained from Toyobo Co., Osaka. Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated. Human non-small cell lung cancer cell lines, PC-9 and PC-14 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. MTT assay was performed as described previously.[4,5] Cells were seeded into 96-well microplates and incubated with a drug for 96 h in the presence or absence of a DNA repair inhibitor, caffeine or aphidicolin. Alkaline elution was performed as described previously.[10] Cells were prelabelled with [methyl-^{14}C]thymidine (0.075 mCi/ml) for 24 h. The labeled cells (5×10^5/dish) were treated with 200 n*M* KW-2189 for 4 h. Then the cells were washed twice with warmed culture medium and incubated in the presence or absence of caffeine (1 m*M*). After the indicated repair period, cells were diluted in cold phosphate-buffered saline (PBS) without Mg^{2+} and Ca^{2+}, gently deposited onto a polycarbonate filter (2.0 μm pore size, 25 mm diameter, Costar Corp., CA) and rinsed with cold PBS. The cells were lysed with 5 ml of lysis solution containing 2% SDS, 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 0.05 ml/min. To analyze the frequency of DNA strand breaks, seven fractions of the eluate were collected directly into scintillation vials at 1.5 h intervals.

In order to ascertain that DNA strand breaks induced by KW-2189 were lethal to cells, we examined the effect of a repair inhibitor on the cytotoxic activity of duocarmycins such as KW-2189, DU-86 and duocarmycin SA. Caffeine was selected as a repair inhibitor because it inhibits poly(ADP ribose) synthesis without affecting unscheduled DNA synthesis induced by UV-irradiation.[11] As shown in Fig. 2, caffeine enhanced the growth-inhibitory activity of the three duocarmycins against PC-9 and PC-14 cells in a dose-dependent manner. No significant effect was observed with another repair modulator, aphidicolin (data not shown).

It is generally recognized that caffeine enhances the cell-killing effect of X-ray irradiation by inhibiting repair of DNA strand breaks. To investigate the role of caffeine in the enhanced growth-inhibitory activity of duocarmycins, the repair kinetics of DNA strand breaks induced by KW-2189 were examined by alkaline elution assay. KW-2189 induced DNA strand breaks in PC-9 cells after a 4 h exposure, while 1 m*M* caffeine did not induce any strand breaks following a 12 h exposure (Fig. 3A). After a 4 h exposure of the cells to KW-2189, cells were washed and incubated for repair in the KW-2189-free medium in the absence or presence of 1 m*M* caffeine (Fig. 3, B and

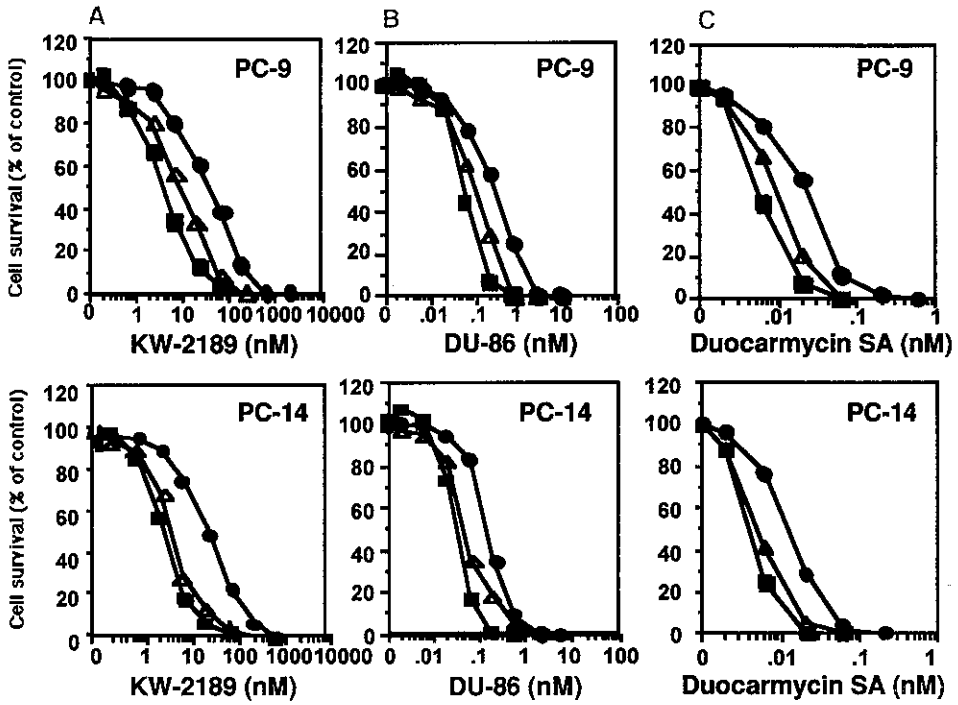

Fig. 2. Effect of caffeine on the cytotoxic activity of duocarmycins in human lung cancer cells. PC-14 cells and PC-9 cells were treated with KW-2189 (A), DU-86 (B), or duocarmycin SA (C) for 96 h in the presence of 0 (●), 0.5 (△) or 1 (■) m*M* caffeine. Cell survival was measured by MTT assay 96 h after the start of drug treatment. Values are the average of three experiments.

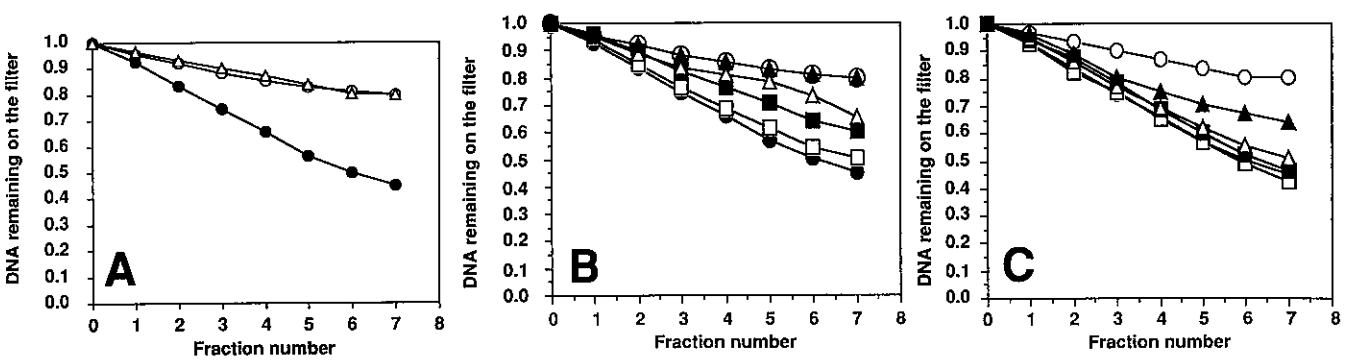

Fig. 3. A, Effect of KW-2189 and caffeine on cellular DNA strand breaks. PC-14 cells were prelabelled with [methyl-^{14}C]-thymidine (0.075 mCi/ml) for 24 h. Cells were treated with 200 n*M* KW-2189 (●) or 1 m*M* caffeine (△) for 4 h and 12 h, respectively. Untreated PC-14 cells were used as a control (○). DNA strand breaks were examined by alkaline elution assay just after treatment. Values are the mean of three experiments. B, C, Effect of caffeine on the repair of DNA strand breaks induced by KW-2189. PC-14 cells were prelabelled with [methyl-^{14}C]thymidine (0.075 mCi/ml) for 24 h. Cells were treated with 200 n*M* KW-2189 for 4 h and washed with warmed medium. Cells were incubated for a further 0 (●), 2 (□), 4 (■), 8 (△) or 12 h (▲) without (B) or with (C) 1 m*M* caffeine. Untreated PC-14 cells with KW-2189 were used as a control (○). Recovered DNA from strand breaks was measured by alkaline elution assay.

C). In the absence of caffeine, DNA repair was observed from 2 h after drug removal and DNA strand breaks were not detected after 12 h of drug-free incubation (Fig. 3B). Previous reports indicated that repairs of DNA strand breaks induced by X-ray irradiation or DNA-cleavage inducible chemicals are usually completed

within 1 or 2 h. The data presented here suggest that DNA strand breaks induced by duocarmycins may be resistant to repair mechanisms. In the presence of caffeine, DNA repair was markedly inhibited until 8 h of KW-2189-free incubation (Fig. 3C). It is probable that DNA strand breaks induced by KW-2189 were potentially lethal to cells and that caffeine enhanced the growth-inhibitory activity of duocarmycins by inhibiting the repair of the DNA strand breaks.

We had previously found that cells treated with KW-2189 showed DNA strand breaks after a lag period of approximately 3 h. Over a 4 h exposure, only 20 n*M* KW-2189 is sufficient to produce DNA strand breaks.[4] In accordance with this, Jacobson *et al.* reported that CC-1065 produced prolonged depletion of the NAD pool in human fibroblasts and xeroderma pigmentosum cells after a lag period of 4 h.[12] Caffeine enhanced the induction of DNA strand breaks by KW-2189. These results suggest that CPI-DNA adducts are not repaired by NER and that the adducts induced DNA strand breaks which required poly(ADP ribose) synthesis for their repair. These results suggested that one of the molecular targets of KW-2189 is involved in NER.

How DNA adducts generate DNA strand breaks is not clear. It might be explained in terms of the general concept that cellular protective responses to genotoxic agents sometimes produce deleterious effects on the cells, such as induction of DNA strand breaks as intermediates of DNA repair. Although we did not investigate whether the DNA strand breaks induced by KW-2189 were repair-associated or not, the inhibitory effect of DNA adducts on NER presumably can cause repair-associated DNA cleavage. Although CC-1065 has been reported not to induce any DNA strand breaks over a 2 h exposure, it is not known whether exposure of the cells to CC-1065 for more than 4 h, the lag period for CC-1065 to produce NAD depletion, could induce DNA strand breaks.

We examined the effect of two repair inhibitors, aphidicolin and caffeine, on the cytotoxic activity of duocarmycins. Our data indicated that caffeine, but not aphidicolin, used at concentrations that did not damage DNA, enhanced the growth-inhibitory activity of duocarmycins by inhibiting the repair of drug-induced DNA cleavage. These results suggested that DNA cleavage induced by KW-2189 is potentially lethal to the cells, and is repaired via a caffeine-sensitive pathway. Sims *et al.* reported that caffeine inhibited the poly(ADP ribose) polymerase activity,[11] but had no effect on unscheduled DNA synthesis induced by UV-irradiation of the cells. It might be that inhibition by caffeine of the repair of DNA strand breaks induced by duocarmycins is due to an inhibitory effect of caffeine on poly(ADP ribose) synthesis. The inhibition of DNA repair by caffeine was more marked at earlier time points than at later time points, suggesting that caffeine inhibited the activities of readily available repair factors such as poly(ADP ribose) polymerase, and that an undetermined damage-inducible repair factor(s) may be affected by caffeine, resulting in decreased cell survival.

In conclusion, our data imply that lethal DNA strand breaks are repaired by a caffeine-sensitive pathway. It is next necessary to identify the repair factor(s) involved. It has already been reported that caffeine enhances the effect of DNA-damaging agents such as cisplatin.[13–15] These results, including ours, suggest that mechanisms of DNA repair are a possible target for anti-cancer chemotherapy, and clinical application of caffeine in combination with DNA-damaging agents may be effective to enhance their antitumor activity.

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

(Received July 7, 1997/Accepted September 2, 1997)

REFERENCES

1) Gomi, K., Kobayashi, E., Miyoshi, K., Ashizawa, T., Okamoto, A., Ogawa, T., Katsumata, S., Mihara, A., Okabe, M. and Hirata, T. Anticellular and antitumor activity of duocarmycins, novel antitumor antibiotics. *Jpn. J. Cancer Res.*, **83**, 113–120 (1992).
2) Kobayashi, E., Okamoto, A., Asada, M., Okabe, M., Nagamura, S., Asai, A., Saito, H., Gomi, K. and Hirata, T. Characteristics of antitumor activity of KW-2189, a novel water-soluble derivative of duocarmycin, against murine and human tumors. *Cancer Res.*, **54**, 2404–2410 (1994).
3) Ogasawara, H., Nishio, K., Takeda, Y., Ohmori, T., Kubota, N., Funayama, Y., Ohira, T., Kuraishi, Y., Isogai, Y. and Saijo, N. A novel antitumor antibiotic, KW-2189 is activated by carboxyl esterase and induces DNA strand breaks in human small cell lung cancer cells. *Jpn. J. Cancer Res.*, **85**, 418–425 (1994).
4) Ogasawara, H., Nishio, K., Kanzawa, F., Lee, Y.-S., Funayama, Y., Ohira, T., Kuraishi, Y., Isogai, Y. and Saijo, N. Intracellular carboxyl esterase activity is a determinant of cellular sensitivity to the antineoplastic agent KW-2189 in cell lines resistant to cisplatin and CPT-11. *Jpn. J. Cancer Res.*, **86**, 124–129 (1995).
5) Boger, D. L., Ishizaki, T. and Zarrinmayeh, H. Synthesis and preliminary evaluation of agents incorporating the pharmacophore of the duocarmycin/pyrindamycin alkylation subunit: identification of the CC-1065/duocarmycin common pharmacophore. *J. Org. Chem.*, **55**, 4499–4502

(1990).
6) Szymkowski, D. E., Yarema, K., Essigmann, J. M., Lippard, S. J. and Wood, R. D. An intrastrand d(GpG) platinum crosslink in duplex M13 DNA is refractory to repair by human cell extracts. *Proc. Natl. Acad. Sci. USA,* **89,** 10772–10776 (1992).
7) Nishikawa, K., Rosenblum, M. G., Newman, R. A., Pandita, T. K., Hittelman, W. N. and Donato, N. J. Resistance of human cervical carcinoma cells to tumor necrosis factor correlates with their increased sensitivity to cisplatin: evidence of a role for DNA repair and epidermal growth factor receptor. *Cancer Res.,* **52,** 4758–4765 (1992).
8) Tang, M. S., Lee, C. S., Doisy, R., Ross, L., Needham-VanDevanter, D. R. and Hurley, L. H. Recognition and repair of the CC-1065-(N3-adenine)-DNA adduct by the UVRABC nucleases. *Biochemistry,* **27,** 893–901 (1988).
9) Tang, M. S., Qian, M. and Pao, A. Formation and repair of antitumor antibiotic CC-1065-induced DNA adducts in the adenine phosphoribosyltransferase and amplified dihydrofolate reductase genes of Chinese hamster ovary cells. *Biochemistry,* **33,** 2726–2732 (1994).
10) Kasahara, K., Fujiwara, Y., Nishio, K., Ohmori, T., Sugimoto, Y., Komiya, K., Matsuda, T. and Saijo, N. Metallothionein content correlates with the sensitivity of human small cell lung cancer cell lines to cisplatin. *Cancer Res.,* **51,** 3237–3242 (1991).
11) Sims, J. L., Sikorski, G. W., Catino, D. M., Berger, S. J. and Berger, N. A. Poly(adenosinediphosphoribose) polymerase inhibitors stimulate unscheduled deoxyribonucleic acid synthesis in normal human lymphocytes. *Biochemistry,* **21,** 1813–1821 (1982).
12) Jacobson, M. K., Twehous, D. and Hurley, L. H. Depletion of nicotinamide adenine dinucleotide in normal and xeroderma pigmentosum fibroblast cells by the antitumor drug CC-1065. *Biochemistry,* **25,** 5929–5932 (1986).
13) Tsuchiya, H., Tomita, K., Yasutake, H., Ueda, Y., Tanaka, M. and Sasaki, T. Growth inhibition and differentiation of murine melanoma B16-BL6 cells caused by the combination of cisplatin and caffeine. *Jpn. J. Cancer Res.,* **80,** 1246–1251 (1989).
14) Tomita, K. and Tsuchiya, H. Caffeine enhancement of the effect of anticancer agents on human sarcoma cells. *Jpn. J. Cancer Res.,* **80,** 83–88 (1989).
15) Yasutake, H., Tsuchiya, H., Tomita, K., Matsunaga, T., Nikaido, O. and Mori, T. Inhibitory effect of caffeine on potentially lethal damage repair in cisplatin-treated human osteosarcoma cells. *Anticancer Res.,* **15,** 831–837 (1995).