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Differential Effect of Duocarmycin A and Its Novel Derivative DU-86 on DNA Strand Breaks in HeLa S_3 Cells

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Duocarmycin A (DUMA) and DU-86, a semisynthetic derivative of duocarmycins (DUMs) and a possible active form of KW-2189, both showed potent cell growth-inhibitory and cell-killing activities against human uterine cervix carcinoma HeLa S_3 cells. Both drugs showed similar profiles of inhibition of macromolecular synthesis and influence on cell-cycle distribution. Namely, they inhibited [^{3}H]thymidine uptake at lower concentrations than [^{3}H]uridine or [^{3}H]leucine uptake, suggesting that the inhibition of DNA synthesis is the primary site of their actions. Furthermore, they induced the accumulation of cells in early S phase. However, a significant difference was observed between these drugs in terms of DNA-fragmentation activity against HeLa S_3 cells by using two independent methods, pulse-field gel electrophoresis and alkaline elution. DNA fragmentation was insignificant in the cells treated with DU-86, in contrast to the cells treated with DUMA. The analysis of DNA adducts in the cells revealed that DU-86 alkylated adenine quite selectively, while DUMA alkylated both adenine and guanine. These results suggest that the pyrrolidone ring of DUMA is responsible for its adduct formation with guanine and the subsequent DNA-fragmentation and inhibition of DNA synthesis, while DU-86 alkylated adenine and inhibited DNA synthesis through mechanisms other than DNA-fragmentation.

Key words: Duocarmycin A — DU-86 — DNA fragmentation

DUMs[4] are antitumor antibiotics isolated from *Streptomyces*[1,2] and possess strong antitumor activity against various tumor cells both *in vitro* and *in vivo*.[3] Among them, duocarmycin A (DUMA) was supposed to be the ultimate active form of DUMs,[3] and was demonstrated to bind covalently to DNA.[4,5] We previously observed remarkable DNA fragmentation in HeLa S_3 cells treated with DUMA and duocarmycins B_1 and B_2 ($DUMB_1$ and $DUMB_2$) by using a contour-clamped homogeneous electric field apparatus.[6] This DNA-fragmentation activity of DUMs was characteristic as compared with those of other antitumor drugs such as intercalators, radical producers and topoisomerase inhibitors.[6] Recently, KW-2189 was synthesized from $DUMB_2$ and was found to possess potent antitumor activity against human and murine transplantable tumors.[7] The cell growth-inhibitory activity of KW-2189 was augmented by incubation with tissue homogenate or esterases, indicating that KW-2189 might be a kind of prodrug which was converted to an active metabolite, DU-86.[7] DU-86-adenine adducts were detected in the DNA of KW-2189-treated cells. However, unlike DUMs, KW-2189 did not cause DNA strand breaks in the cells.[7] It was also reported that DNA strand breaks were not detected in cells treated with CC-1065[8] or adozelesin (U-73,975),[9] in spite of their structural similarities to DUMA. To elucidate these differences in DNA-fragmentation activity among DUMs, KW-2189 and CC-1065 analogs, we conducted a comparative study of DUMA and DU-86. The only structural difference between DUMA and DU-86 is in the A-ring, where pyrrolidone in DUMA is replaced by pyrrole in DU-86 (Fig. 1). This structural difference is involved in the "common pharmacophore" of a duocarmycin/CC-1065 alkylation subunit,[10,11] and the structure of CC-1065 is similar to that of DU-86. In this report, we show that the difference in A-ring structure between DU-86 and DUMA is responsible for their different DNA-fragmentation activity at the cellular level, although their effects on DNA synthesis exhibit strong similarities.

MATERIALS AND METHODS

Chemicals Adriamycin and mitomycin C were obtained from the authors' company. DUMA was produced and purified as reported previously.[2] DU-86 was chemically synthesized from $DUMB_2$ as an A-ring pyrrole analog.[12]

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[4] Abbreviations: DUM, duocarmycin; EDTA, ethylenediaminetetraacetic acid; EDTA-2Na, ethylenediaminetetraacetic acid disodium salt; IC_{50}, concentration required for 50% growth inhibition; IC_{90}, concentration required for 90% cell-killing; kbp, kilobase pairs; PBS(−), Dulbecco's phosphate-buffered saline (Ca^{2+}-, Mg^{2+}-free, pH 7.2).

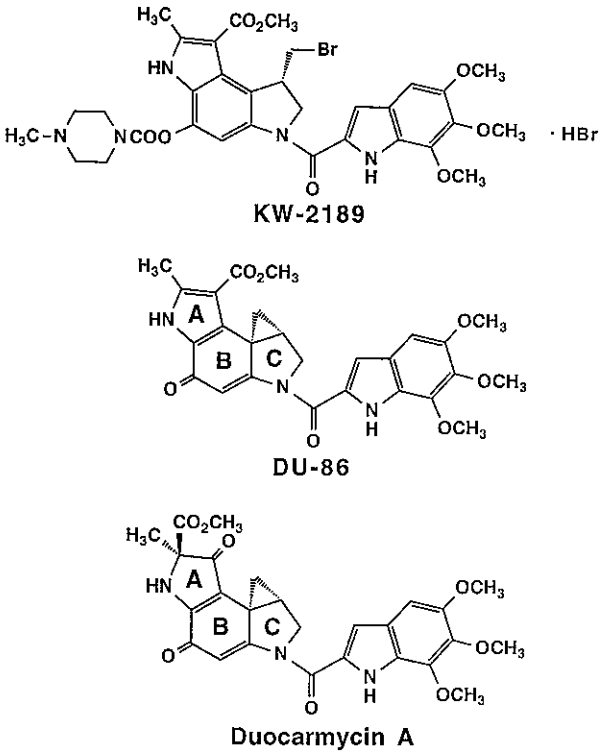

Fig. 1. Structures of KW-2189, DU-86 and DUMA.

Cell culture Human uterine cervix carcinoma HeLa S_3 cell line was purchased from American Type Culture Collection through Dainihon Pharmaceutical Co. (Osaka). The cells were cultured in monolayer in Eagle's minimal essential medium (MEM, Nissui Pharmaceutical Co., Tokyo) supplemented with 10% fetal bovine serum (Grand Island Biological Co., Grand Island, NY), hereafter designated as culture medium, at 37°C under a humidified atmosphere containing 5% CO_2 in air.

Growth-inhibitory activity HeLa S_3 cells (2×10^4/well) were precultured for 24 h in 24-well multidishes (Nunc, Roskilde, Denmark) containing 0.75 ml of culture medium as described above. Then the cells were treated with each compound for 1 h, washed with PBS(−) and further incubated for 71 h in culture medium. The cell number was counted in a Toa micro-cell counter (Toa Medical Electronics Co., Hyogo) according to the method previously reported.[13] Cell growth-inhibitory activity of compounds was compared in terms of IC_{50} values. Values are the mean of three independent experiments.

Cell-killing activity Cell-killing activity was determined in terms of inhibition of colony formation of HeLa S_3 cells. Cells were seeded at 150, 750, 3800 or 15000 cells/well in 6-well tissue culture clusters (Costar, Cambridge, MA), incubated for 3 h, and treated with each compound for 1 h. Then the cells were washed with PBS(−) and further incubated in culture medium for 7 days. Colonies were stained with crystal violet solution (0.05% (w/v) crystal violet (Wako Pure Chem. Indust., Osaka), 20% (v/v) ethanol and 7% (v/v) formaldehyde) and counted. Values are mean±SD of three independent experiments. Cell-killing activity of compounds was compared in terms of IC_{90} values.

Macromolecular synthesis HeLa S_3 cells (2×10^4/well) were precultured for 24 h in 24-well multidishes (Nunc) containing 0.5 ml of culture medium, and treated with each compound. At the indicated time, 2.5 μCi/ml of [^{3}H]thymidine (DuPont/NEN Research Products, Boston, MA), [^{3}H]uridine or [^{3}H]leucine (Amersham Japan Co., Tokyo) was added, and incubation was continued at 37°C for 60 min. The culture supernatant was discarded, then the monolayer cells were washed twice with ice-cold PBS(−), and treated with 5% (w/v) ice-cold trichloroacetic acid (Wako) solution for 20 min. The acid-insoluble fraction was dissolved in 0.5 ml of 1 *N* NaOH at 37°C for 12–16 h, and radioactivity was measured with a liquid scintillation counter. Values are the mean±SD of three independent experiments.

Cell cycle analysis HeLa S_3 cells (6×10^5/dish) were precultured for 24 h in a plastic dish (Nunc) containing 20 ml of culture medium. Then the cells were treated with each compound, and at the indicated time, they were washed with PBS(−), detached with 0.02% (w/v) EDTA-2Na solution, fixed with 70% ethanol solution, hydrolyzed with 25 μg/ml of ribonuclease A (type 1-A, Sigma Chemical Co., St. Louis, MO) at 37°C for 30 min, and stained with propidium iodide (Sigma). The cells were analyzed with a Coulter EPICS C cell sorter (Coulter Electronics, Inc., Hialeah, FL) to evaluate the cell cycle distribution.

DNA extraction and pulse field gel electrophoresis HeLa S_3 cells (9×10^6/flask) were precultured for 24 h in plastic flasks (Nunc) containing 30 ml of culture medium. After 2-h treatment with each compound, the cells were washed with PBS(−), detached with 0.02% EDTA-2Na solution, and centrifuged. The cell pellet was suspended in 50 m*M* EDTA-2Na (pH 8.0), mixed with an equal volume of 1.0% (w/v) low-temperature-melting agarose (Japan Bio-Rad Co., Tokyo), placed in a sample-plug caster (Japan Bio-Rad Co.), and left at 4°C for 60 min to solidify.[3] The cells in the agarose block were treated with lysis buffer (10 m*M* Tris-base, 500 m*M* EDTA-2Na, 1% (v/v) Sarkosyl and 1 mg/ml proteinase K (Boehringer Mannheim Yamanouchi Co., Tokyo), pH 8.0) at 50°C for 48 h and washed three times with 50 m*M* EDTA-2Na for 3 days at 4°C. Plugs about 3 mm long were cut from the agarose blocks and loaded onto 1.0% (w/v) agarose gel (agarose H, Wako) in electrophoresis

buffer (45 m*M* Tris-base, 45 m*M* boric acid, 1.25 m*M* EDTA-2Na, pH 8.3). Electrophoresis was carried out by using a contour-clamped homogeneous electric field apparatus (horizontal gel chamber, a model 200/20 power supply and Pulsewave 760 switcher; Japan Bio-Rad Co.) at 10–15°C in electrophoresis buffer. The run time was 15 h at 6 V/cm (200 V) with a 60 s switch time followed by 8 h with 90 s switch time at a reorientation angle of 120 degrees. DNA size standards prepared from yeast chromosomes (Japan Bio-Rad Co.) were used as markers. Gels were stained overnight in the dark with 1.5 μg/ml of ethidium bromide (Sigma), destained in deionized water, and photographed on a UV light box with Polaroid 667 positive-negative film.

Alkaline elution HeLa S_3 cells (4×10^4/dish) precultured for 24 h in plastic dishes (Nunc) were labeled with [^{14}C]thymidine (0.1 μCi/ml, DuPont/NEN) for 24 h, washed with PBS(−), and further incubated in culture medium for 24 h. Then the cells were treated with drugs at 37°C for 1 h, washed once with ice-cold PBS(−), detached with 0.02% EDTA-2Na solution on ice, and collected on a polycarbonate filter (pore size, 2.0 μm, 25 mm diameter; Nuclepore Corp., Pleasanton, CA). These cells were treated with proteinase K (0.5 mg/ml; Boehringer Mannheim Yamanouchi Co.) in the presence of 2% (w/v) sodium lauryl sulfate (Wako) and 25 m*M* EDTA solution (pH 9.6) for 1 h at room temperature in the dark to digest DNA-bound protein. The filter was washed twice with 3 ml of 20 m*M* EDTA solution (pH 10.0), and DNA was eluted with tetrapropylammonium hydroxide (Eastman Kodak, Rochester, NY), containing 20 m*M* EDTA (pH 11.9) at a rate of 0.05 ml/min. Fractions were collected directly into scintillation vials at intervals of 90 min. The radioactivity of each fraction was measured and expressed as a proportion of total radioactivity.

DNA adduct DNA adducts in DU-86- or DUMA-treated cells were analyzed as follows. HeLa S_3 cells (1.2×10^7/dish) were precultured for 24 h in plastic culture dishes (Nunc) containing 30 ml of culture medium. After 12-h treatment with 20 μM test compound, the cells were washed with PBS(−), detached with 0.02% (w/v) EDTA-2Na solution, and centrifuged. The cell pellet was treated with 0.5% NP-40 solution for 10 min on ice, washed with sterile 0.9% NaCl solution, and treated with lysis buffer (10 m*M* Tris-base, 1 m*M* EDTA-2Na, 1% (v/v) Sarkosyl, and 1 mg/ml proteinase K, pH 8.0) at 47°C for 24 h with shaking. DNA was purified by phenol extraction and ethanol precipitation, and this step was repeated after RNase (Boehringer Mannheim Yamanouchi Co.) treatment at 36°C for 1 h. The DNA purity was considered acceptable when the ratio of absorbance at 260 nm and 280 nm was over 1.8. These DNA samples were dissolved in 10 m*M* phosphate buffer (pH 7.0) at the concentration of 1 mg/ml, heated at 100°C for 10 min for depurination, and extracted with ethyl acetate. The organic layer was concentrated and analyzed by reverse-phase HPLC (wavelength, 330 nm; column, Unisil $_5C_{18}$-250A; elution solution, 40% acetonitrile/50 m*M* potassium phosphate buffer (pH 5.9); flow rate, 1 ml/min). The retention time of purine-drug conjugates was compared with those of the authentic DU-86-adenine, DU-86-guanine, DUMA-adenine and DUMA-guanine adducts, which were obtained from 50 mg of calf thymus DNA treated with DU-86 (5 mg) or DUMA (5 mg) in 200 ml of phosphate buffer (pH 7.0) and prepared according to the above methods.

RESULTS

Growth-inhibitory activity and cell-killing activity of DU-86 and DUMA against HeLa S_3 cells In advance of the analysis of DNA fragmentation, growth-inhibitory activity of DU-86 and DUMA against HeLa S_3 cells was examined (Fig. 2). The IC_{50} values of DU-86 and DUMA were 0.23 n*M* and 0.12 n*M*, respectively, and the activity of these drugs was about 1×10^3 times more potent than that of mitomycin C or adriamycin. Based on these IC_{50} values, suitable concentrations of each compound were chosen for further examinations.

Cell-killing activity of DU-86 and DUMA against HeLa S_3 cells was examined by colony-formation assay (Fig. 3). DU-86 or DUMA showed cell-killing activity in a log-linear dose-response manner over a range of at least

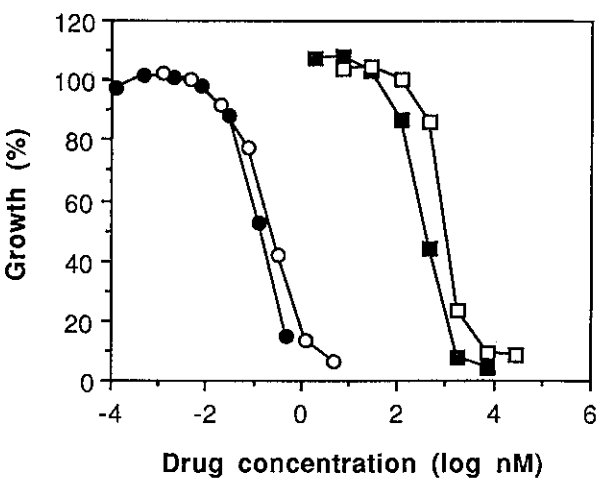

Fig. 2. Growth-inhibitory activity of DU-86, DUMA, adriamycin and mitomycin C against HeLa S_3 cells. The cells (2×10^4/well) were precultured for 24 h, treated with various concentrations of DU-86 (○), DUMA (●), adriamycin (■) or mitomycin C (□) for 1 h on day 1, and further incubated for 71 h in culture medium.

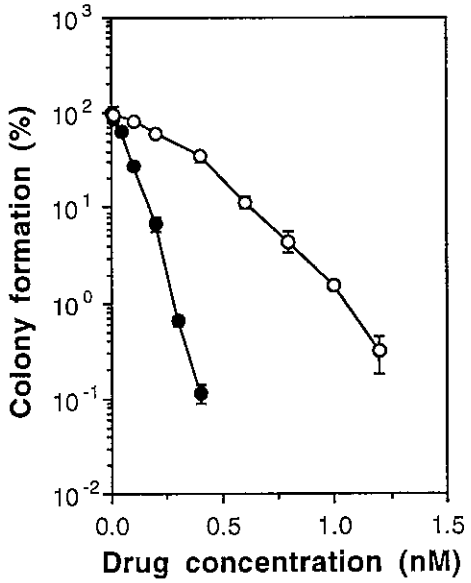

Fig. 3. Cell-killing activity of DU-86 or DUMA against HeLa S_3 cells. The cells (150, 750, 3800 and 15000/well) were precultured for 3 h, treated with various concentrations of DU-86 (○) or DUMA (●) for 1 h, and further incubated for 7 days in culture medium. Cell-killing activity was determined in terms of colony formation. Relative plating efficiency of control cells was 49%.

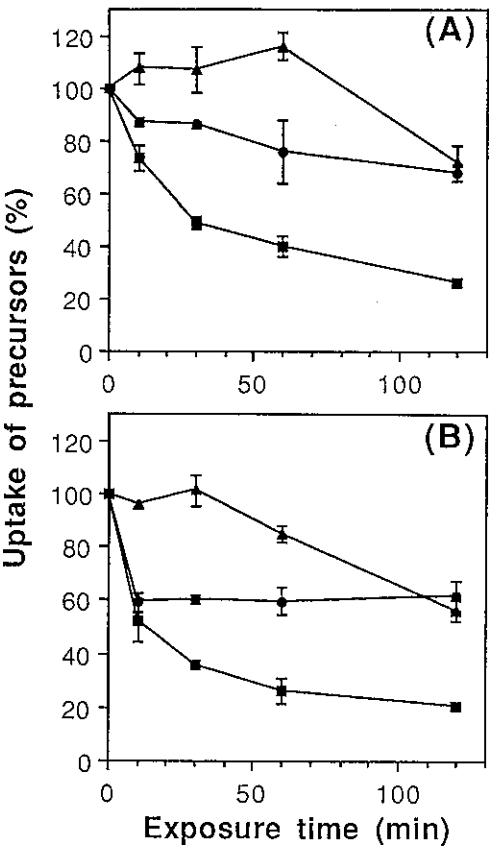

Fig. 4. Inhibition of macromolecular synthesis by DU-86 and DUMA. HeLa S_3 cells (2×10^4/well) were precultured for 24 h and treated with 2.0 n*M* DU-86 (A) or 2.0 n*M* DUMA (B). At the indicated time, 2.5 μCi/ml of [^{3}H]thymidine (■), [^{3}H]uridine (●) or [^{3}H]leucine (▲) was added to the medium, and the incorporation into macromolecules was determined as described in "Materials and Methods."

2 logs. The IC_{90} values, 0.63 n*M* for DU-86 and 0.17 n*M* for DUMA, indicate that the cell-killing activity of each compound was manifested at relatively similar concentration to that required for cell growth inhibition.

Effects of DU-86 and DUMA on macromolecular synthesis and cell cycle distribution To investigate the action sites of DU-86 and DUMA, their effects on DNA, RNA and protein synthesis were examined (Fig. 4). In the case of DU-86-treated cells, the incorporation of [^{3}H]thymidine into macromolecules was most strongly inhibited at short exposure time as compared with that of [^{3}H]uridine or [^{3}H]leucine, and this inhibition increased exposure time-dependently (Fig. 4A). Similar results were observed in DUMA-treated cells (Fig. 4B). The IC_{50} values of DU-86 and DUMA for incorporation of [^{3}H]thymidine were about 0.8 n*M* and 0.2 n*M*, and for those with [^{3}H]uridine were 5.3 n*M* and 2.4 n*M*, respectively. From these data, it was indicated that the primary action of DU-86 and DUMA on macromolecular synthesis was the inhibition of DNA synthesis.

The DNA histogram of HeLa S_3 cells treated with DU-86 or DUMA was examined to compare their effects on the cell cycle distribution (Fig. 5). At 0.63 n*M* DU-86 or 0.17 n*M* DUMA, the IC_{90} values for cell-killing activity, the cell population in the G2M phase decreased and the cells accumulated in early S phase at 8 h after treatment. This effect of DU-86 lasted longer than that of DUMA, suggesting that the DNA modification by DU-86 is more irreversible.

Comparison of DNA fragmentation in HeLa S_3 cells To investigate the effect of DU-86 and DUMA on cellular DNA, HeLa S_3 cells were treated with these drugs, and DNA was extracted and analyzed by pulse-field gel electrophoresis using a contour-clamped homogeneous electric field apparatus (Fig. 6). This technic is sensitive enough to separate DNA as large as yeast chromosomal DNA (2200–245 kbp). The DNA of untreated cells was so large that it did not migrate in the running gel (1.0% agarose) under these conditions (lane 2). However, DNA fragmentation occurred in the cells treated with 1.2 n*M* DUMA (lane 4), 10 times its IC_{50} value. After treatment with 12 n*M* DUMA, DNA was fragmented to a size between 1800 and 245 kbp (lane 5), and at 120 n*M*

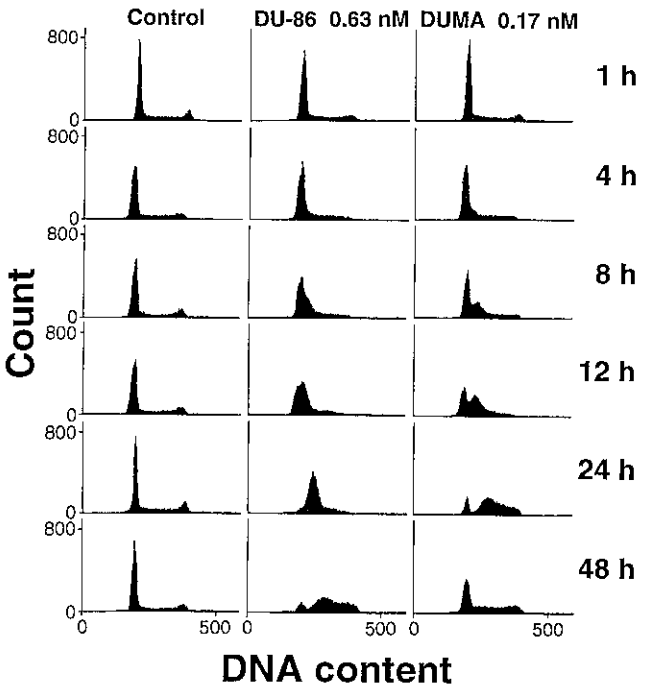

Fig. 5. Effect of DU-86 and DUMA on cell cycle distribution of HeLa S_3 cells. The cells (6×10^5/dish) were precultured for 24 h and treated with 0.63 n*M* DU-86 or 0.17 n*M* DUMA for the indicated time. The DNA histogram was analyzed by flow cytometry as described in "Materials and Methods."

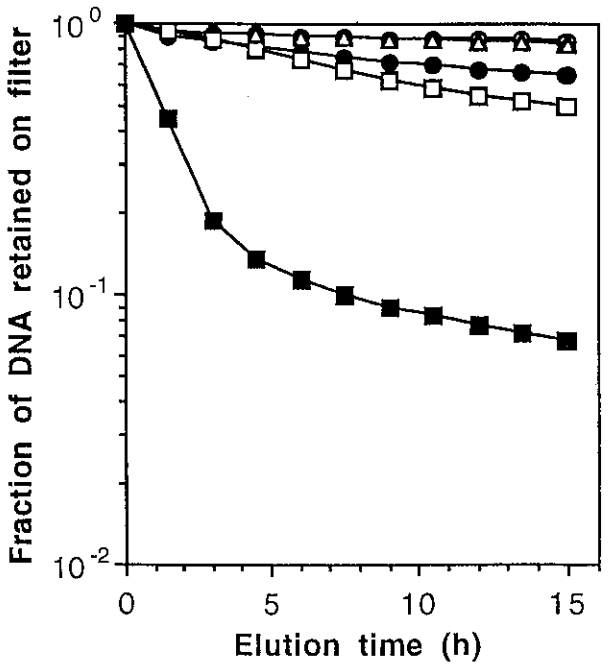

Fig. 7. Elution pattern of DNA of HeLa S_3 cells analyzed by alkaline elution. The cells (4×10^4/dish) were precultured for 24 h, labeled with [^{14}C]thymidine for 24 h, and further incubated for 24 h in culture medium. The cells were then treated for 1 h with 23 n*M* DU-86 (△), 1.2 n*M* DUMA (□), 12 n*M* DUMA (■), 1.7 μM adriamycin (●) and untreated (○), and subjected to alkaline elution as described in "Materials and Methods."

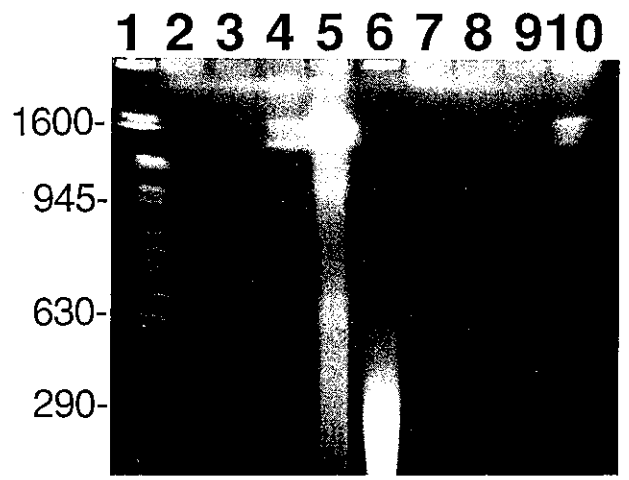

Fig. 6. Pulse-field gel electrophoresis of DNA extracted and purified from DUMA- or DU-86-treated HeLa S_3 cells. The cells (9×10^6/flask) were precultured for 24 h and treated with DUMA or DU-86 for 2 h. The DNA extraction and agarose gel electrophoresis on the contour-clamped homogeneous electric field apparatus were conducted as described in "Materials and Methods." Lanes: 1, DNA size standard (kbp): 2, untreated; 3–6, DUMA 0.12, 1.2, 12 and 120 n*M*; 7–10, DU-86 0.23, 2.3, 23 and 230 n*M*.

DUMA, DNA fragments below 600 kbp were observed (lane 6). On the contrary, DNA fragmentation was not detected in the cells treated with 23 n*M* DU-86, 100 times its IC_{50} value (lane 9). Since this agarose gel electrophoresis was applied for analysis of DNA fragments under neutral conditions, DNA fragments induced by double-strand breaks were supposed to be detected.

To verify the above results, DNA in the cells treated with DU-86 or DUMA was analyzed by the alkaline elution method, which can detect single-strand breaks of DNA. DNA in the cells treated with 1.7 μM adriamycin, 10 times its IC_{50} value at 1-h exposure, was used as a positive control (Fig. 7). DNA in the cells treated with 1.2 n*M* DUMA, 10 times its IC_{50} value, eluted more rapidly than that treated with 1.7 μM adriamycin, and at 12 n*M* DUMA, the elution rate of DNA was remarkable. On the contrary, the elution pattern of DNA in the cells treated with 23 n*M* DU-86, 100 times its IC_{50} value, was quite similar to that in untreated cells, indicating that DU-86 did not cause DNA fragmentation in the cells, even in a single-strand break mode, under the condition of 1-h exposure.

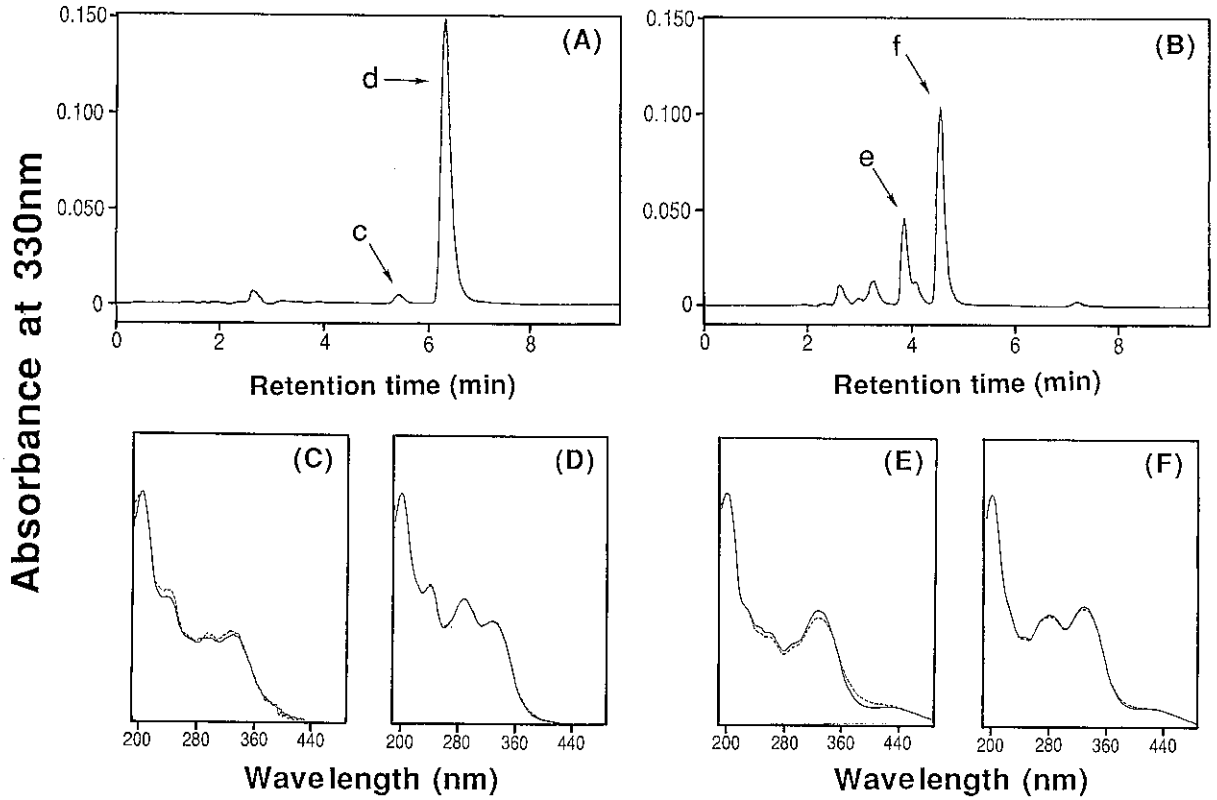

Fig. 8. DNA adducts detected in HeLa S_3 cells treated with DU-86 or DUMA. The cells (1.2×10^7/dish) were precultured for 24 h and treated with 20 μM DU-86 (A) or DUMA (B) for 12 h. DNA was extracted and adducts were analyzed by HPLC as described in "Materials and Methods." Peaks of c, d, e, and f (retention time; 5.4 min, 6.3 min, 3.8 min, 4.5 min, respectively) were identified as DU-86-guanine (C), DU-86-adenine (D), DUMA-guanine (E), and DUMA-adenine (F) adducts, respectively (dotted line). The authentic drug-DNA adducts were obtained from calf thymus DNA treated with DU-86 or DUMA (C–F, solid line).

DNA adducts From the results in Figs. 4, 6 and 7, the inhibition of DNA synthesis by DU-86 was suggested to be induced by mechanisms other than DNA-fragmentation. To elucidate the intrinsic action mechanisms of DU-86 and DUMA, DNA adducts in HeLa S_3 cells treated with DU-86 or DUMA were analyzed. For standard drug-DNA adduct samples, calf thymus DNA was treated with DU-86 or DUMA, and drug-DNA adducts were purified by TLC and identified by NMR (data not shown). The absorbance spectrum of DNA adducts detected in DU-86- or DUMA-treated cells was compared with that of standard drug-DNA adduct samples (Fig. 8C–F). The major peak detected in DNA extracted from DU-86-treated cells (Fig. 8A; peak d) corresponded to DU-86-adenine adducts (Fig. 8D), and a minor peak (Fig. 8A; peak c) to DU-86-guanine adducts (Fig. 8C). In contrast, two major peaks were detected and corresponded to DUMA-adenine adducts (Fig. 8B; peak f) and DUMA-guanine adducts (Fig. 8B; peak e). The ratios of adenine adducts and guanine adducts were 37:1 for DU-86 and 2.5:1 for DUMA. The components in the other minor peaks detected in DNA extracted from DU-86- or DUMA-treated cells were not identified. These differences in DNA adduct profile between DU-86- and DUMA-treated cells are supposed to be associated with their different DNA-fragmenting effects.

DISCUSSION

We previously reported that duocarmycins A, B_1 and B_2, which possess potent cytotoxic and antitumor activities,[3] induced significant DNA fragmentation in HeLa S_3 cells.[6] In this report, we investigated the DNA-fragmenting activity of DU-86, which is supposed to be an active form of KW-2189,[7] compared with that of DUMA. The difference in their structure is just that the pyrrole ring in DU-86 replaces the pyrrolidone ring in DUMA. Both DU-86 and DUMA showed quite similar

characteristics of potent cell growth-inhibitory activity (Fig. 2), cell-killing activity (Fig. 3), inhibition of macromolecule synthesis (Fig. 4), and the accumulation in early S phase of the cell cycle distribution (Fig. 5) in HeLa S_3 cells. However, unexpectedly, the DNA-fragmenting activities of these drugs were found to be quite different; namely, no DNA fragmentation was detected in DU-86-treated cells by pulse-field gel electrophoresis, whereas fragmentation was detected in DUMA-treated cells (Fig. 6). This difference in DNA-fragmenting activities was confirmed by the alkaline elution method (Fig. 7). In a cell-free system, DUMA caused DNA strand breaks directly in plasmid DNA pBR322, and as a result, supercoiled DNA was converted to circular DNA after treatment with DUMA at 37°C for 24 h. However, open-circular DNA was not detected even after treatment with DU-86, indicating that DNA strand breaks were not induced by DU-86 in this system (data not shown). Ogasawara *et al.* observed DNA strand breaks in KW-2189-treated NCI-H69 cells by the alkaline elution method.[14] This DNA fragmentation was observed after treating cells with KW-2189 for more than 3 h. DU-86 may induce DNA fragmentation to some extent at longer exposure times in our system.

It was reported that CC-1065 analogs, which possess a similar structure to DUMA, did not cause fragmentation of cellular DNA.[8,9] Our result with DU-86 is thus similar to that of CC-1065 analogs. DU-86 and CC-1065 possess a pyrrole ring within the "common pharmacophore,"[10,11] whereas DUMA possesses a pyrrolidone ring. Thus, the pyrrolidone ring of DUMA appears to be essential for DNA-fragmenting activity. DU-86 and DUMA showed different base specificity of DNA alkylation; namely, DU-86 alkylated adenine quite selectively (Fig. 8A), as was also reported for CC-1065,[15] whereas DUMA alkylated both adenine and guanine (Fig. 8B). The structural difference, pyrrole in DU-86 and pyrrolidone in DUMA, could account for their base selectivity of DNA alkylation, leading to DNA fragmentation only in DUMA-treated cells. DUMA was reported to induce DNA alkylation followed by a depurination reaction, which might cause a DNA strand break.[10] DUMA could alkylate adenine and guanine of DNA, and depurination of them could cause strong DNA fragmentation. In spite of these differences in DNA-fragmenting activity between DU-86 and DUMA, they exhibited similar characteristics in terms of the inhibition of macromolecular synthesis (Fig. 4) and effect on cell-cycle kinetics (Fig. 5). DNA was suggested to be the primary site of action of these drugs (Fig. 4), and a delay of the cell cycle in the S-phase was induced by these drugs (Fig. 5). CC-1065 was also reported to inhibit the synthesis of cellular DNA,[16] although it did not cause DNA fragmentation.[8] Moreover, analysis of cell cycle kinetics showed that accumulation of cells in the S-phase was induced by the treatment with CC-1065[17] or adozelesin.[18,19] It was reported that CC-1065 inhibited the activities of DNA helicase,[20,21] polymerase[22] and ligase,[23] and these actions of CC-1065 might be deeply associated with its anticellular activity. DU-86 is also suggested to cause inhibition of DNA synthesis via interference with these DNA-directed enzymes, leading to potent anticellular activity. Further investigations are progressing.

In conclusion, the pyrrolidone ring of DUMA is responsible for the adduct formation with guanine, and subsequent DNA-fragmentation and inhibition of DNA synthesis, while DU-86 forms adducts with adenine selectively and inhibits DNA synthesis through mechanisms other than DNA fragmentation.

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