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Anticellular and Antitumor Activity of Duocarmycins, Novel Antitumor Antibiotics

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The anticellular and antitumor activities of novel antitumor antibiotics, duocarmycins (DUMs), were examined against human and murine tumor cells. DUMs consist of five compounds, A, B_1, B_2, C_1 and C_2, which possess a pharmacophore similar to that of CC-1065, a previously isolated antibiotic. Among them, DUMA exhibited ultrapotent growth-inhibitory activity with an IC_{50} value of 6 p*M* against human uterine cervix carcinoma HeLa S_3 cells. DUMA and DUMB1 also inhibited the growth of adriamycin (ADM)-resistant lines of human nasopharynx carcinoma KB cells and breast carcinoma MCF-7 cells as well as their sensitive lines. DUMs inhibited the growth of s.c.-inoculated murine tumors such as B16 melanoma, sarcoma 180, M5076 sarcoma and colon 26. DUMs were also significantly effective in increasing the lifespan of i.p.-inoculated B16 melanoma-bearing mice, although their effect was marginal against other i.p.-inoculated tumors. As a whole, DUMB1 exhibited superior activity to the other four compounds. DUMB1 rapidly inhibited the incorporation of [^{3}H]-TdR into macromolecules of HeLa S_3 cells as compared with that of [^{3}H]UR or [^{3}H]leucine. DNA strand breaks were detected in DUMB1-treated HeLa S_3 cells by agarose gel electrophoresis with a contour-clamped homogeneous electric field apparatus. These results indicate that DUMs possess interesting biological activities as DNA-targeting antitumor antibiotics.

Key words: Antitumor activity — Antibiotic — Duocarmycin — DNA strand break

Duocarmycins (DUMs) were isolated from *Streptomyces* sp. as novel antitumor antibiotics with potent anticellular activity.[1-4] So far, five related compounds have been discovered and named duocarmycin A (DUMA), B_1 (DUMB1), B_2 (DUMB2), C_1 (DUMC1) and C_2 (DUMC2). Firstly DUMA, DUMC1 and DUMC2 were isolated from the fermentation broth,[1-3] and recently DUMB1 and DUMB2 were isolated from the fermentation broth of the same microorganism after modification of the medium composition.[4] Their structures are shown in Fig. 1. All five compounds exhibited *in vivo* antitumor activity almost equal to that of mitomycin C (MMC) against s.c.-inoculated murine sarcoma 180.[4] DUMB1, DUMB2, DUMC1 and DUMC2 were supposed to be prodrugs of DUMA.

CC-1065 is an antitumor antibiotic that was discovered and characterized in The Upjohn Company.[5,6] This compound is one of the most cytotoxic antitumor agents known, with IC_{50} values of p*M* order,[7,8] and it was active against several experimental murine tumors *in vivo*.[6] Since then, many analogs have been synthesized and their biological activities reported.[9] The action mechanism of these compounds was reported to be minor groove-binding to DNA and subsequent alkylation of the N3 position of adenine, resulting in the cleavage of DNA strands.[10]

The structural similarity of CC-1065 and DUMs suggested the existence of a common pharmacophore, which has recently been confirmed.[11] The present study was carried out in order to investigate the influence of structural difference of the pharmacophore of DUMs on the *in vitro* anticellular activity and *in vivo* antitumor activity.

Abbreviations: ADM, adriamycin; CHEF, contour-clamped homogeneous electric field; DUM, duocarmycin; IC_{50}, concentration required for 50% growth inhibition; MMC, mitomycin C; MTD, maximum-tolerated dose; PBS (−), Dulbecco's phosphate-buffered saline (Ca^{2+}-, Mg^{2+}-free).

MATERIALS AND METHODS

***In vitro* growth-inhibitory activity** Preculture of various cell lines, i.e., uterine cervix carcinoma HeLa S_3, human nasopharynx carcinoma KB and its adriamycin (ADM)-resistant line (KB-A1)[12] in Eagle's minimal essential medium (MEM, Nissui Pharmaceutical Co., Tokyo) containing 10% fetal bovine serum (Grand Island Biological Co., Grand Island, N.Y.), or human breast carcinoma MCF-7 and its ADM-resistant line (MCF-7/ADM)[13] in RPMI-1640 medium containing 10% fetal bovine serum, 100 units of penicillin and 100 μg/ml of streptomycin (Grand Island Biological Co.), was conducted at the concentration of 2.5×10^4 cells/well for 24 h in 24-well multidishes (Nunc, Roskilde, Denmark) containing 0.75 ml of culture medium at 37°C in a humidified atmosphere containing 5% CO_2 in air. Then HeLa S_3 cells were treated with each compound for 1 h,

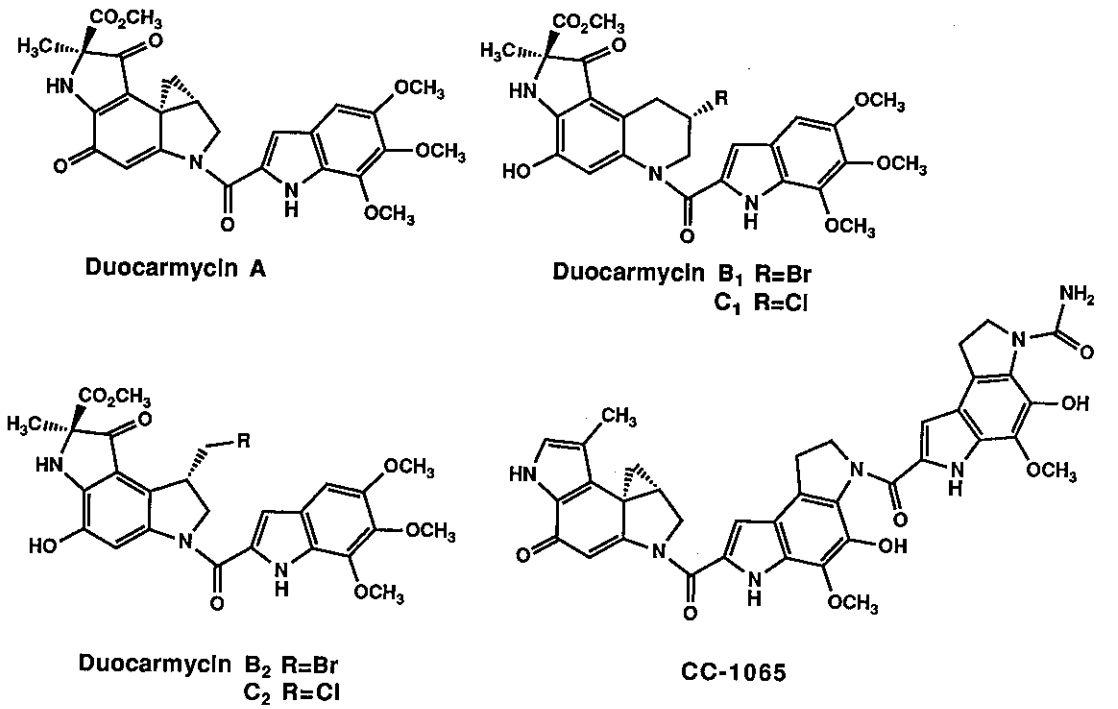

Fig. 1. Structures of DUMs and CC-1065.

washed, and further incubated for 71 h in the culture medium. Other cell lines were treated with each compound continuously for 72 h. The cell number was counted by using a Toa micro-cell counter (Toa Medical Electronics Co., Ltd., Hyogo) according to the method previously reported.[14] Growth-inhibitory activity was expressed as IC_{50} value.

Macromolecular synthesis The effect of DUMs on DNA, RNA and protein synthesis was determined as follows. HeLa S_3 cells (2×10^4/well) in 0.75 ml of culture medium were precultured in 24-well multidishes. After 24 h, the cells were treated with each compound and incubated at 37°C. At the indicated time, 2.5 μCi/ml of [^{3}H]TdR, [^{3}H]UR or [^{3}H]leucine (Amersham Japan Co., Tokyo) was added, and further incubated at 37°C for 60 min. Then the culture supernatant was discarded, and the monolayer cells were washed twice with ice-cold Dulbecco's phosphate-buffered saline (PBS(−), Ca^{2+}-, Mg^{2+}-free, pH 7.2) and treated with 5% ice-cold trichloroacetic acid (Wako Pure Chemical Industries, Ltd., Osaka) 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 the radioactivity was measured with a liquid scintillation counter.

DNA preparation and electrophoresis HeLa S_3 cells (9×10^6/30 ml culture medium) were precultured in plastic flasks (NUNC). After 24 h, the cells were treated with each compound at 37°C for 2 h. Then the culture supernatant was discarded, and the monolayer cells were washed with ice-cold PBS(−), treated with 0.02% (w/v) disodium EDTA, and centrifuged. The cell pellet was suspended in PBS(−) and mixed with an equal volume of 1.0% (w/v) low temperature-melting agarose (Japan Bio Rad Co., Tokyo). The cells in agarose were treated with lysis buffer (10 m*M* Tris-base, 500 m*M* disodium EDTA, 1% (w/v) Sarkosyl, 1 mg/ml proteinase K, pH 8.0) at 50°C for 48 h, and washed three times with 50 m*M* disodium EDTA for three days. These DNA preparations were loaded onto 1.0% (w/v) agarose gel in electrophoresis buffer (45 m*M* Tris-base, 45 m*M* boric acid, 1.25 m*M* disodium EDTA, pH 8.3), and the electrophoresis was performed by using a contour-clamped homogeneous electric field (CHEF) apparatus (Japan Bio Rad Co.) at 10–15°C for 8 or 24 h. DNA size standards prepared from yeast chromosomes (Japan Bio Rad Co.) were used as markers. Gels were stained in the dark with 8 μg/ml of ethidium bromide (Sigma Chemical Co., St. Louis, Mo.) for 1 h, destained overnight in deionized water, and photographed on an ultraviolet light box.

Animals and tumors Sarcoma 180 cells were passaged and used for the experiment in adult male ddY mice weighing 19–21 g. Murine lymphocytic leukemia P388 and lymphoid leukemia L1210 cells were passaged in

adult male DBA/2 mice and used for the experiment in adult male BALB/c×DBA/2 F_1 (CDF_1) mice weighing 20–25 g. M5076 reticulum cell sarcoma and B16 melanoma cells were passaged and used in adult male C57BL/6 mice weighing 20–25 g. Colon adenocarcinoma 26 cells were passaged and used in adult male BALB/c mice weighing 20–25 g. These animals were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka). Sarcoma 180 was kindly supplied by the National Cancer Center (Tokyo), and P388 leukemia, L1210 leukemia, M5076 reticulum cell sarcoma, B16 melanoma and colon adenocarcinoma 26 by the Japanese Foundation for Cancer Research (Tokyo). All animal experiments were conducted with 5 mice in a group.

Evaluation of antitumor activity Murine solid tumors were inoculated s.c. at the axillary region of mice. The length and width of tumors were measured, and their volume was calculated by using the following formula according to the method of the National Cancer Institute[15]:

$$\text{Tumor volume (mm}^3\text{)} = \frac{\text{length (mm)} \times [\text{width (mm)}]^2}{2}$$

The criteria for effectiveness were a treated versus control value (percent) of 50 or less, and a statistically significant difference as determined by the Mann-Whitney U test.

The antitumor activity against i.p.-inoculated tumors was evaluated by calculating the percentage increase in life span.

RESULTS

Growth-inhibitory activity of DUMs The growth-inhibitory activity of DUMs against human uterine cervix carcinoma HeLa S_3 cells was compared with that of MMC (Fig. 2). All compounds inhibited the growth of HeLa S_3 cells dose-dependently. The IC_{50} values after 1 h exposure were 6 p*M* for DUMA; 35 p*M* for DUMB-1; 100 p*M* for DUMB2; 570 p*M* for DUMC2; and 8500 p*M* for DUMC1. The activity of DUMA or DUMB1 was about 1×10^5 times more potent than that of MMC, of which the IC_{50} value was 1.0 μM.

The existence of tumor cells resistant to multiple chemotherapeutic agents including ADM is a major problem during cancer chemotherapy.[12, 13, 16] The new antibiotics may be candidates for drugs which can overcome multidrug resistance. Therefore the growth-inhibitory activity of DUMs against ADM-resistant tumor cell lines was examined (Table I). KB-Al and MCF-7/ADM cells exhibited resistance ratios of 210 and 31 to ADM, respectively, indicating that these cell lines retained the characteristics of multidrug resistance. The

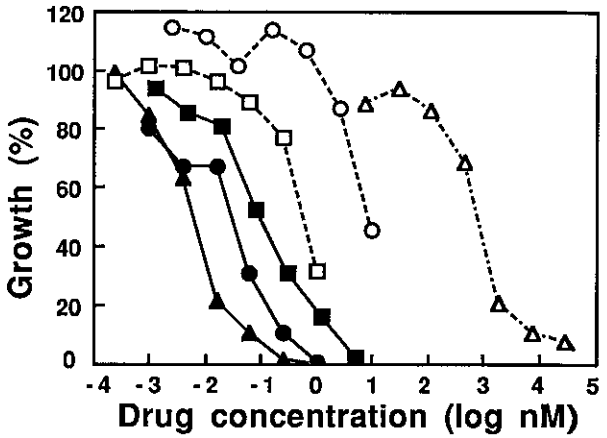

Fig. 2. Growth-inhibitory activity of DUMs against human HeLa S_3 cells. The cells (2.5×10^4/well) were cultured on day 0 and treated with various concentrations of DUMA (▲), DUMB1 (●), DUMB2 (■), DUMC1 (○), DUMC2 (□), or MMC (△) for 1 h on day 1, and then further incubated for 71 h in the drug-free medium.

Table I. Growth-inhibitory Activity of DUMA and DUMB1 against ADM-resistant Human Tumor Cell Lines[a]

Compounds	IC_{50} (n*M*)		Resistance ratio[b]
	KB cells	KB-Al cells	
DUMA	0.064	0.12	1.9
DUMB1	0.027	0.032	1.2
MMC	24	530	22
ADM	11	2300	210
	MCF-7 cells	MCF-7/ADM cells	
DUMA	0.034	0.027	0.79
DUMB1	0.0046	0.0045	0.98
MMC	58	94	1.6
ADM	15	470	31

a) The cells (2.5×10^4/well) were cultured on day 0 and treated with the compounds on day 1 for 72 h. On day 4, the anticellular activity was determined as described in "Materials and Methods."
b) Ratio of IC_{50} value of KB-A1 or MCF-7/ADM cells to that of KB or MCF-7 cells, respectively.

IC_{50} values of DUMA and DUMB1 against both ADM-resistant cell lines were almost equal to those against parent sensitive cell lines, respectively, indicating that DUMs were effective in inhibiting the growth of these resistant cell lines.

Antitumor activity of DUMs The LD_{10} values of DUMA, DUMB1, DUMB2 and DUMC1 by single i.v.

Table II. Antitumor Activity of DUMs against i.p.-Inoculated Murine B16 Melanoma[a]

Exp. No.	Groups	Schedule	Dose (mg/kg/day)	Mean survival days±SD	ILSmax[b] (%)
1	Untreated		0	11.7±0.7	0
	DUMB1	Day 1	0.063	22.2±1.9[d]	90
		Day 1, 5, 9	0.031	24.6±2.2[d]	110
		Day 1–5	0.016	21.6±4.8[d]	85
	DUMB2	Day 1	0.031	19.8±3.4[d]	69
	DUMC1	Day 1	1.0	23.2±3.0[d]	98
	MMC	Day 1	4.0	>31.0±16.2[d]	>165 (1/5)[c]
2	Untreated		0	13.0±1.0	0
	DUMA	Day 1	0.0039	18.4±0.9[d]	42
	DUMB1	Day 1	0.063	30.0±10.0[d]	131
	DUMC2	Day 1	0.13	21.8±0.5[d]	68

a) B16 (0.5 ml/mouse of 10% homogenate) cells were inoculated i.p. on day 0. Compounds were administered i.p. on each day.
b) ILSmax, maximal increase in life span.
c) Survivor for more than 60 days.
d) ILSmax (%)≧25, and $P<0.05$ by Mann-Whitney U test, as compared with the control group.

Table III. Antitumor Activity of DUMs against s.c.-Inoculated Murine B16 Melanoma[a]

Groups	Dose (mg/kg/day)	Schedule	Tumor volume (mm^3, mean±SD)	T/C[b] (%)
Untreated	0		1797±710	100
DUMA	0.050	Day 1, 5, 9	473±143[c]	26
DUMB1	0.25	Day 1, 5, 9	886±204[c]	49
DUMB2	0.25	Day 1, 5, 9	632±549[c]	35
DUMC1	5.8	Day 1, 5, 9	523±147[c]	29
MMC	6.0	Day 1, 5, 9	108±61[c]	6.0

a) B16 (0.1 ml/mouse of 20% homogenate) cells were inoculated s.c. on day 0. Compounds were administered i.v. on day 1, 5, 9. Tumor volume was measured on day 17.
b) T/C, treated versus control.
c) T/C(%)≦50, and $P<0.05$ by Mann-Whitney U test, as compared with the control group.

treatment were 0.027, 0.33, 0.25 and 11.8 mg/kg, respectively, in male ddY mice. Considering these LD_{10} values, the antitumor activity of DUMs was examined at several doses (2-fold dilution), and the results at MTD on each treatment schedule are shown in the table. The body weight decrease in DUM-treated mice was moderate for an antitumor drug (data not shown). First of all, the antitumor activity of DUMs was compared in i.p.-inoculated murine B16 melanoma-bearing mice, since this tumor was more sensitive to DUMs (Table II). Among them, DUMB1 and DUMC1 showed more potent activity upon single treatment, and DUMB1 and DUMB2 were effective at lower dose as compared with DUMC1. The schedule dependency of the antitumor activity was tested by using the typical compound DUMB1, which was a little more effective in intermittent treatment than single treatment. Therefore the antitumor activity of DUMs against s.c.-inoculated murine tumors was examined by using intermittent treatment, except for sarcoma 180, which grew too fast for intermittent treatment to be feasible (Tables III and IV). DUMA, DUMB-1, DUMB2 and DUMC1 were also effective at inhibiting the growth of s.c.-inoculated murine B16 melanoma (Table III). The further examinations in murine solid tumor-bearing mice indicated that the antitumor activity of DUMB1 and DUMB2 was equal to or more than that of DUMA, DUMC1 or DUMC2. Therefore only the results for DUMB1 and DUMB2 are summarized in Table IV. The efficacy of DUMB1 was almost equal to that of DUMB2 in solid sarcoma 180-, M5076-, or colon 26-bearing mice. In i.p.-inoculated P388 leukemia-, L1210 leukemia- or M5076 sarcoma-bearing mice, the increase of life span of mice treated with DUMB1 was a little more than that of mice treated with DUMB2 (Table V). As a whole, the antitumor activity of DUMB1 was considered to be most potent among the five compounds.

Inhibition of macromolecular synthesis by DUMs To elucidate the mechanisms of the anticellular activity of DUMs, their effect on DNA, RNA and protein synthesis was examined in HeLa S_3 cells (Fig. 3). For this experiment, DUMB1 was chosen because of its potent *in vitro* growth-inhibitory activity (Fig. 2) and more significant *in vivo* antitumor activity (Tables II–V). The incorporation of [^{3}H]TdR into macromolecules of the cells was inhibited biphasically by DUMB1. Namely the inhibition

Table IV. Antitumor Activity of DUMB1 and DUMB2 against s.c.-Inoculated Murine Tumors[a]

Tumors	Groups	Dose (mg/kg/day)	Tumor volume (mm^3, mean±SD)	T/C[b] (%)	On day
Sarcoma 180	Untreated	0	3518±360	100	7
	DUMB1	0.25	1270±551[c]	36	7
	DUMB2	0.25	1163±245[c]	33	7
	MMC	6.0	670±273[c]	19	7
M5076	Untreated	0	568±198	100	12
	DUMB1	0.25	221±26[c]	39	12
	DUMB2	0.25	161±58[c]	28	12
	MMC	6.0	239±20	42	12
Colon 26	Untreated	0	1499±121	100	12
	DUMB1	0.25	131±47[c]	8.7	12
	DUMB2	0.13	121±92[c]	8.1	12
	MMC	6.0	322±118[c]	21	12

a) Sarcoma 180 (5×10^6/mouse), M5076 (1×10^6/mouse) and colon 26 (0.1 ml/mouse of 20% homogenate) cells were inoculated s.c. on day 0. DUMB1 and DUMB2 were administered i.v. on day 1 into sarcoma 180-bearing mice or on days 1, 5 and 9 into M5076- and colon 26-bearing mice. MMC was administered i.v. on day 1.
b) T/C, treated versus control.
c) T/C(%)$\leqq$50, and $P<0.05$ by Mann-Whitney U test, as compared with the control group.

Table V. Antitumor Activity of DUMB1 and DUMB2 against i.p.-Inoculated Murine Tumors[a]

Tumors	Groups	Dose (mg/kg)	Mean survival days±SD	ILSmax[b] (%)
P388	Untreated	0	10.6±0.7	0
	DUMB1	0.016	13.4±1.5[c]	26
	DUMB2	0.0078	12.4±0.9	17
	MMC	4.0	16.0±0.2[c]	51
L1210	Untreated	0	8.6±0.5	0
	DUMB1	0.016	10.8±3.3	26
	DUMB2	0.0078	9.8±1.6	14
	MMC	4.0	12.4±1.7[c]	44
M5076	Untreated	0	17.1±1.6	0
	DUMB1	0.031	22.4±1.5[c]	31
	DUMB2	0.031	20.2±4.0	18
	MMC	6.0	26.0±2.9[c]	52

a) P388 and M5076 (1×10^6/mouse), and L1210 (1×10^5/mouse) cells were inoculated i.p. on day 0. Compounds were administered i.p. on day 1.
b) ILSmax, maximal increase in life span.
c) ILSmax(%) $\geqq$25, and $P<0.05$ by Mann-Whitney U test, as compared with the control group.

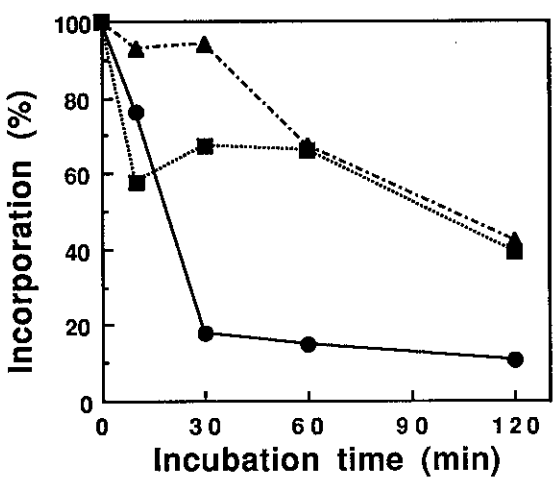

Fig. 3. Effect of DUMB1 on the incorporation of labeled precursors into DNA, RNA and proteins. HeLa S_3 cells (2×10^4/well) were precultured for 24 h and treated with 50 n*M* DUMB1. At the indicated time, 2.5 μCi/ml of [^{3}H]TdR (●), [^{3}H]UR (■) or [^{3}H]leucine (▲) was added to the medium, and the incorporation into the macromolecules was determined as described in "Materials and Methods."

rate increased significantly at the exposure time between 15 and 30 min, and then maintained an almost constant level, suggesting the reaction of DUMB1 with DNA was rapid. On the other hand, the inhibition of the incorporation of [^{3}H]UR or [^{3}H]leucine by DUMB1 was insignificant.

DNA strand breaks CC-1065, which possesses a pharmacophore similar to that of DUMs, was reported to bind covalently to N3 of adenine, resulting in breakage of DNA strands.[10] Therefore we analyzed the size of DNA extracted from HeLa S_3 cells treated with DUMB1 by agarose gel electrophoresis using a CHEF apparatus.

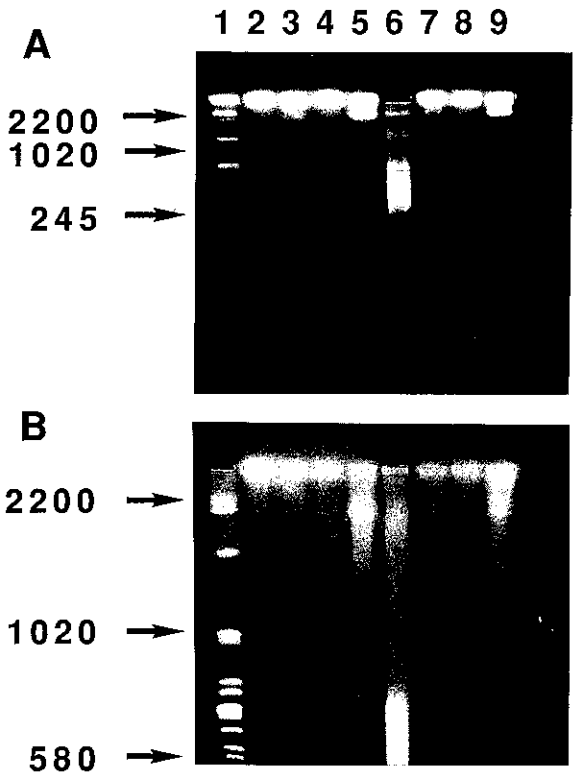

Fig. 4. Agarose gel electrophoresis of DNA extracted and purified from DUMB1- or MMC-treated HeLa S_3 cells. HeLa S_3 cells (9×10^6/flask) were precultured in plastic flasks for 24 h and treated with DUMB1 or MMC for 2 h. The DNA extraction and agarose gel electrophoresis on the CHEF apparatus were described in "Materials and Methods." The same DNA samples were run for 8 h (A) or 24 h (B). Lanes: 1, DNA size standard (kilobases); 2, untreated; 3, DUMB1 35 p*M*; 4, DUMB1 350 p*M*; 5, DUMB1 3.5 n*M*; 6, DUMB1 35 n*M*; 7, MMC, 10 μM; 8, MMC 100 μM; 9, MMC 1 m*M*.

This apparatus enabled us to analyze DNA as large as yeast chromosomal DNA. We applied this apparatus for the analysis of ultralarge DNA extracted from the drug-treated HeLa S_3 cells (Fig. 4). The DNA of untreated cells was so large that it did not move in 1.0% agarose gel under these conditions at all (lane 2). However after the treatment of the cells with DNA-targeting drugs as DUMB1 or MMC, DNA strand breaks became detectable by the CHEF apparatus. This could be seen more clearly at longer running time of the electrophoresis (24 h rather than 8 h). DUMB1 caused DNA strand breaks to the size of about 1600 kb at 3.5 n*M*, 100 times its IC_{50} value (lane 5). At 35 n*M*, the DNA was cleaved to the size range between 300 and 800 kb (lane 6). On the other hand, MMC did not cause DNA strand breaks at 100 μM, 100 times its IC_{50} value (lane 8), and caused slight breaks to the size over 1600 kb at 1 m*M* (lane 9). These results indicate that the DNA-cleaving activity of DUMB1 was at least 1×10^5 times as potent as that of MMC.

DISCUSSION

CC-1065 is one of the most cytotoxic antitumor agents known, with IC_{50} values of p*M* order.[7,8] DUMs also exhibited highly potent growth-inhibitory activity against HeLa S_3 cells even at the exposure time of 1 h (Fig. 2). Among them, DUMA was the most potent and its IC_{50} value was 6 p*M*. The electrophilic cyclopropane present commonly in CC-1065 and DUMA was demonstrated to induce N3 alkylation of adenine of DNA strands.[10,11] The most potent growth-inhibitory activity of DUMA among the five related compounds may be attributed to its cyclopropane structure (Fig. 1). The halogens of DUMB1, DUMB2, DUMC1 and DUMC2 are supposed to be cleaved in the culture medium or the cells, resulting in the conversion of these compounds to DUMA, which is the ultimate anticellular agent. Therefore these four compounds were thought to be prodrugs of DUMA, and the difference of their growth-inhibitory activity might be explained in terms of their intrinsic conversion rates to DUMA in the culture medium or the cells.

In *in vivo* experiment, DUMs were effective at inhibiting the growth of s.c.-inoculated murine tumors such as sarcoma 180, B16 melanoma, M5076 sarcoma and colon 26 (Tables III and IV). The structural difference between DUMB1 or DUMB2 and DUMC1 is the species of halogen acting as a leaving group (Fig. 1). DUMB1 and DUMB2 contain bromine, whereas DUMC1 contains chlorine, and this difference might be related to their differential toxicity in mice. Namely the MTD values of DUMB1 and DUMB2 were 0.25 mg/kg/day, whereas that of DUMC1 was 5.8 mg/kg/day (Table III), suggesting bromine-containing compounds have more potent activity *in vivo*. Cisplatin, which is a typical alkylating agent with superior antitumor activity clinically, has chlorines as a leaving group. The platinum analog with bromines as a leaving group was also synthesized. However, its *in vivo* antitumor activity and toxicity in mice were less than that of cisplatin.[17] Bromine may have an important role in the *in vivo* antitumor activity of DUMB-1 and DUMB2. Although the development of CC-1065 was stopped because of the delayed death of mice at subtherapeutic doses,[18] DUMs did not have such an effect in mice (data not shown).

The existence of tumor cells resistant to chemotherapeutic agents is a major problem during chemotherapy.[16] KB-Al and MCF-7/ADM cells showed acquired resistance to multiple agents including ADM.[12,13] DUMA and DUMB1 inhibited the growth of these resistant cells as well as their parent sensitive lines (Table I), suggesting

DUMs may be useful for overcoming multidrug resistance. Multidrug resistance has been explained in terms of the expression of multidrug-resistance genes, resulting in the enhanced efflux of drugs from the tumor cells and the decrease of their intracellular concentration. DUMs were suggested to have negligible affinity for multidrug-resistance proteins, resulting in the accumulation of DUMs in the ADM-resistant cell lines to the same degree as in the sensitive cell lines. Further investigations are desirable in this regard.

The mechanism of antitumor activity of CC-1065 was reported to be minor groove-binding to DNA and subsequent alkylation of the N3 position of adenine, resulting in the cleavage of DNA strands.[10] However these experiments were performed in an *in vitro* cell-free system, and the effect on DNA strands of intact cells remains undetermined. The structural similarity of the pharmacophores of CC-1065 and DUMs led us to analyze the size of DNA strands extracted from the DUMB1-treated HeLa S_3 cells (Fig. 4). The CHEF apparatus enabled us to analyze DNA as large as yeast chromosomal DNA by agarose gel electrophoresis.[19] We applied this apparatus for the analysis of ultralarge DNA of mammalian cells. As expected, DNA from untreated HeLa S_3 cells did not move in the agarose gel, because its size is so large even as compared with that of yeast chromosomal DNA. On the other hand, DNA prepared from DUMB1-treated cells moved clearly in the agarose gel. DUMB1 caused DNA strand breaks to the size of 1600 kb at 3.5 n*M*, 100 times its IC_{50} value. MMC did not cause DNA strand breaks at 100 μM, 100 times its IC_{50} value, but caused slight breaks over 1600 kb at 1 m*M*. The primary mechanism of the anticellular activity of MMC was reported to be the alkylation of DNA strands,[20] which might not be detected as the DNA strand breaks by CHEF. However at higher concentration, MMC was reported to generate oxygen radicals and induce DNA strand breaks.[21, 22] The slight but clear DNA strand breaks detected by CHEF in MMC-treated HeLa S_3 cells might be explained by the latter action mechanism of MMC. In any cases, these results indicate that CHEF may be useful to analyze DNA strand breaks at the cellular level, and the primary mechanism of antitumor activity of DUMB1 is suggested to be DNA strand breakage.

In conclusion, DUMs possess interesting antitumor activity against murine solid tumors, and appear to act at the cellular DNA level.

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