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ORIGINAL ARTICLE

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A novel synthetic DNA minor groove binder, MS-247: antitumor activity and cytotoxic mechanism

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Abstract *Purpose*: MS-247 is a novel synthetic compound possessing a DNA-binding moiety and a DNA-alkylating residue, chlorambucil. In this study, we evaluated the antitumor activity of MS-247 against murine tumor cell lines and its effects on DNA molecules in both cell-free and cellular systems. *Methods*: The in vitro cytotoxic activity of MS-247 was evaluated against four murine tumor cell lines, P388, L1210, Colon26 and B16, and its in vivo antitumor activity was also tested in comparison with Adriamycin (ADM), cisplatin (CDDP) and paclitaxel. The ability of MS-247 to associate with the DNA minor groove was assessed by measuring quenching of Hoechst 33342 fluorescence. DNA-DNA interstrand crosslinks (ICL) were detected by an alkaline elution assay for cellular DNA and a band-shift assay using the plasmid pBR322. The effects of MS-247 on macromolecule synthesis (DNA, RNA and proteins) were examined by measuring incorporation of the radiolabeled precursors. *Results*: MS-247 exhibited in vitro cytotoxicity with IC_{50} values ranging 11 to 500 n*M*, and MS-247 given i.v. showed strong in vivo antitumor activity against i.p.-implanted L1210 leukemia cells and s.c.-implanted Colon26 carcinoma cells, and moderate activity against i.p.-implanted P388 leukemia cells but no apparent activity against s.c.-implanted B16 melanoma cells. MS-247 reversibly displaced Hoechst 33342 bound to DNA within a few minutes, and irreversibly formed ICL within 1–6 h in both the cell-free system and the cellular system. These results suggest that an association of MS-247 with the DNA minor groove occurred more quickly than ICL formation. The inhibition of DNA synthesis was more prominent than the inhibition of RNA and protein synthesis in L1210 cells exposed to MS-247, and a 6-h incubation with MS-247, which formed apparent ICL in the cellular system, strongly inhibited DNA synthesis. This result suggests that impairment of DNA replication preceded the inhibition of RNA and protein synthesis and that ICL formation greatly contributed to the inhibition of macromolecule synthesis. *Conclusion*: The results of this study suggest that MS-247 exerts its cytotoxic effect through impairment of DNA function by getting into the minor groove of DNA and subsequently forming ICL. MS-247 has potent antitumor activity with a different spectrum from the activity of clinically proven antitumor agents such as paclitaxel, ADM and CDDP against several murine tumor cell lines. This result suggests that MS-247 may be useful for the treatment of human cancers.

Key words MS-247 · DNA minor groove binder · DNA-DNA interstrand crosslinks · Antitumor effect · Netropsin

Abbreviations *ADM* Adriamycin · *CDDP* cis-diamminedichloroplatinum(II) · IC_{50} concentration required for a 50% inhibition of cell growth · *ICL* DNA-DNA interstrand crosslinks · *ILS* increase in lifespan · *MTD* maximum tolerated dose · *MTT* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide · *PBS(−)* calcium- and magnesium-free phosphate-buffered saline · *SDS* sodium dodecyl sulfate

Introduction

DNA-targeting agents have been used for cancer therapy for more than 50 years and are still used extensively as one of the most important classes of antitumor agents. They can be divided into the following three classes: (1) compounds that are able to bind covalently to DNA such as the alkylating agent chlorambucil and

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the DNA crosslinker CDDP [19]; (2) compounds that lead to DNA breakage such as the bleomycins [20]; and (3) compounds that bind reversibly to double-helical DNA such as the intercalators anthracyclines and the minor groove-binder netropsin [2]. Since the antibiotic agent CC-1065 has been reported to have potential anticancer activity [15], interest in agents that show both class 1 and class 2 mechanisms has arisen [3, 23].

We have attempted to synthesize compounds possessing both DNA-bialkylating and minor groove-binding functions, similar to the extensively studied compounds tallimustine and CC-1065 analogs, which have broad antitumor activity against human tumors [6, 13, 18]. We selected a DNA minor groove-binding moiety (Fig. 1, compound 1) by assays based on inhibition of DNA digestion with restriction enzymes and inhibition of cell growth from our compound library designed in accordance with spatial and electrical fitting to the curvature of minor groove of B-type DNA. Thereafter, we tested various alkylating groups as partners of compound 1 for their ability to provide effective inhibition of cell growth. Finally, MS-247 (Fig. 1) was selected as one of the most potent agents against Colon26 murine solid tumors in vivo. Therefore, MS-247 consists of two parts, compound 1 (Fig. 1) which is expected to selectively bind AT-rich regions of the DNA minor groove and chlorambucil (Fig. 1) which binds covalently with DNA [10, 19]. Here, we report a series of studies concerning the mechanism of action, in vitro cytotoxicity and in vivo antitumor activity of MS-247 against murine tumors.

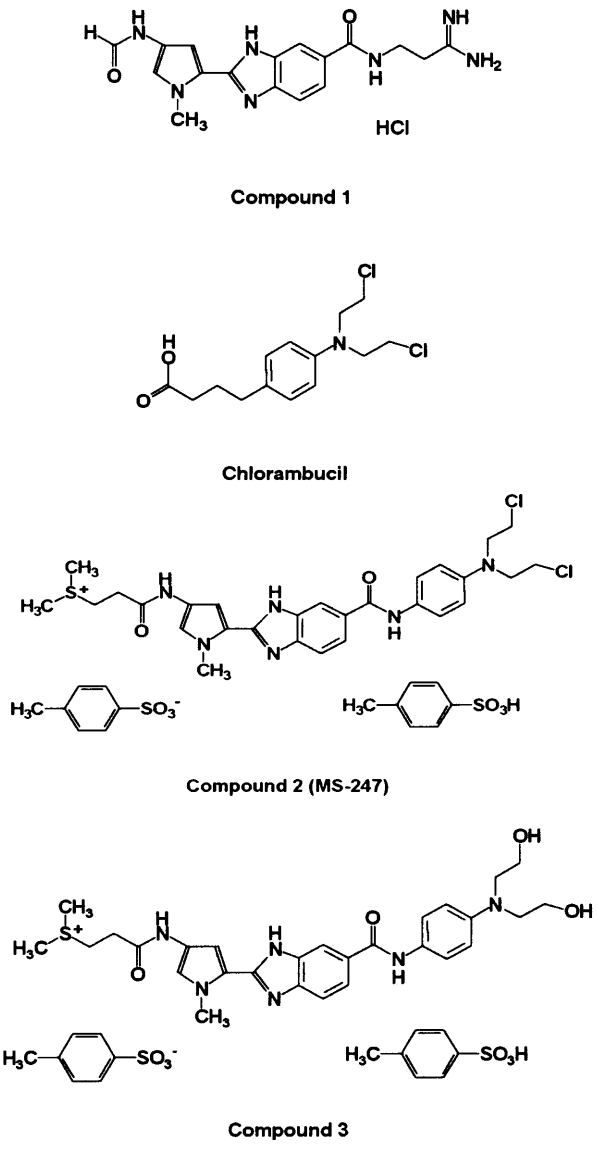

Fig. 1 Chemical structures of MS-247 and related compounds

Materials and methods

Chemicals

MS-247 and compound 3 were synthesized as described previously [16]. Chlorambucil, Hoechst 33342, Adriamycin (ADM), cisplatin (CDDP) and paclitaxel were purchased from Sigma Chemical Co. (St. Louis, Mo.), Molecular Probes (Eugene, Ore.), Kyowa Hakko Kogyo Co. (Tokyo, Japan), Nippon Kayaku Co. (Tokyo, Japan), and Biolyse Co. (Port-Daniel, Quebec, Canada), respectively. MS-247, compound 3 and chlorambucil were dissolved in *N*,*N*-dimethylformamide immediately before use. [2-^{14}C]Thymidine (57.0 mCi/mmol), [5-^{3}H]uridine (27.0 Ci/mmol), and L-[4,5-^{3}H] leucine (157 Ci/mmol) were purchased from Amersham Pharmacia Biotech (Tokyo, Japan).

Animals and tumor cell lines

Female CDF_1, BDF_1, BALB/c, DBA/2 and C57BL/6 mice (6 weeks old) were purchased from Japan SLC (Shizuoka, Japan) and used at 7 weeks of age under specific pathogen-free conditions. The murine leukemia cell lines, L1210 and P388, the murine colorectal carcinoma cell line, Colon26, and the murine melanoma cell line, B16, were obtained from the Japanese Foundation for Cancer Research (Tokyo, Japan). For in vitro studies, the cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 0.15% $NaHCO_3$, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μ*M* 2-mercaptoethanol at 37 °C in humidified air containing 5% CO_2. For in vivo experiments, P388 and L1210 cells were passaged in DBA/2 mouse ascites, and Colon26 and B16 cells were passaged in the axillary region of BALB/c and C57BL/6 mice, respectively.

In vitro growth inhibition assay

Tumor cells were seeded in 96-well plates at a density of 1000 cells/well (P388 and L1210) or 750 cells/well (B16 and Colon26) in 150 μl culture medium, and after a 24-h culture, the cells were treated with MS-247 (0.0001–100 μg/ml) for 72 h. Cell growth was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [1]. Growth inhibition was expressed as the ratio of absorbance of the drug-treated wells to that of control wells, and the concentrations that resulted in 50% growth inhibition (IC_{50}) were determined.

Evaluation of antitumor activity in vivo

P388 leukemia cells (1×10^6) and L1210 leukemia cells (1×10^5) were implanted i.p. into CDF_1 mice. Colon26 cells (1×10^6) and B16 cells (1×10^6) were implanted s.c. into CDF_1 mice and BDF_1 mice, respectively. On day 1 after implantation, MS-247, ADM or

CDDP was administered i.v. once. Paclitaxel was administered i.v. daily on days 1–5. MS-247 was dissolved in a vehicle comprising 5% (w/v) Tween 80 in 5% (w/v) dextrose solution. ADM and CDDP were dissolved in saline. Paclitaxel was dissolved in a vehicle comprising 5% (w/v) ethanol, 5% (w/v) cremophor EL in 5% (w/v) dextrose solution.

For ascites tumors, the antitumor activity of the drugs was estimated by determining the increase in lifespan (ILS). The percentage ILS was calculated from the formula: ILS(%) = $[(T/C) - 1] \times 100$, where T and C indicate the median survival period of the treated group and control group, respectively.

For solid tumors, tumor weight (g) was measured on day 15. Antitumor activity was determined by calculating the ratio of the mean tumor weight of the treated group to that of the control group. Statistical significance was estimated by the Steel test ($P < 0.001$ or $P < 0.01$). All in vivo experiments were conducted with six mice per group.

Displacement assay

The association of MS-247 with the minor groove of DNA was detected by measuring the quenching of Hoechst 33342 fluorescence [21] as a result of replacement with MS-247 in both cell-free and cellular assay systems. For cell-free experiments, 1 μg calf thymus DNA (Sigma, St. Louis, Mo.) in 0.2 ml calcium- and magnesium-free phosphate-buffered saline [PBS(−)] was preincubated with 6.1 μ*M* Hoechst 33342 for 10 min at room temperature in 96-well plates. The test compound was then added followed by a further 10-min incubation. Fluorescence was measured by a fluorometer (Fluoroskan II; Labsystems, Helsinki, Finland) at an excitation wavelength of 355 nm and emission wavelength of 460 nm.

For cellular experiments, L1210 cells (4×10^5) were preincubated with 6.1 μ*M* Hoechst 33342 in 1 ml culture medium at 37 °C for 20 min. The test compound was then added followed by a further 20-min incubation at 37 °C. Cells were washed once with chilled PBS(−), and subsequently resuspended in chilled PBS(−) and the fluorescence was measured as described above.

Alkaline elution assay

Alkaline elution assay was performed according to the method of Kohn et al. [12] with some modifications. Cells (1×10^5/ml) were prelabeled with 0.1 μCi/ml [^{14}C]thymidine for 24 h at 37 °C, and were treated with 20 μ*M* MS-247 for 2 or 6 h. The cells were deposited onto a polycarbonate filter (2.0 μm pore size, 25 mm diameter; Millipore Corporation, Bedford, Mass.) and rinsed twice with PBS(−). The cells were lysed with 5 ml lysis solution comprising 2% SDS, 25 m*M* disodium EDTA, 50 m*M* Tris, 50 m*M* glycine and 0.5 mg/ml proteinase K (pH 10.0) for 1 h at room temperature in the dark. Then, the filter was rinsed with 3 ml 20 m*M* EDTA (pH 10.0), and irradiated by UV lamp (GL15, Toshiba Co., Tokyo, Japan) for 2 min at a range of 2.5 cm. DNA was eluted with 30 ml 20 m*M* EDTA-tetrapropylammonium hydroxide (pH 12.1) containing 0.1% SDS at a flow rate of 0.03 ml/min in the dark, and the fractions (2.7 ml/1.5 h per tube) were collected for 15 h. The radioactivities of the fractions were measured using a liquid scintillation counter (1600TR; Packard Instrument Company) after the addition of ACS II (Amersham Pharmacia Biotech, Tokyo, Japan).

Detection of ICL in plasmid DNA

The plasmid DNA pBR322 (Boehringer Mannheim, Tokyo, Japan) was linearized by digestion with Hind III (Toyobo Co., Osaka, Japan), and incubated with MS-247 or CDDP in 15 μl of a solution comprising 10 m*M* Tris-HCl (pH 8.0) and 1 m*M* EDTA at 37 °C. After incubation for 15 min, 1 h and 2 h, an aliquot (4 μl) of the reaction mixture was mixed with 8 μl 20 m*M* NaOH, 1 m*M* EDTA, 4.6% Ficoll, and 0.25% bromocresol green, and electrophoresed in an alkaline agarose gel containing 0.9% agarose, 33 m*M* NaOH and 2 m*M* EDTA. DNA was visualized by staining with ethidium bromide after neutralization of the gel with 30 m*M* Tris-HCl (pH 7.5).

Determination of macromolecule synthesis

L1210 cells (3×10^4/well) were preincubated for 5 h in 96-well plates, and MS-247 diluted with culture medium, which had been kept at 37 °C, was added. After incubation for 10, 100 and 340 min, 18 nCi/ml [^{14}C]thymidine, 0.36 μCi/ml [^{3}H]uridine or 0.71 μCi/ml [^{3}H]leucine was added followed by incubation for a further 20 min. The cells were harvested onto GF/C filters (Skatron, Tranby, Norway) using a cell harvester (type 11025; Skatron), and the filter was treated with 10% (w/v) trichloroacetic acid and then washed with ethanol. The radioactive precipitates on the filter were measured using a liquid scintillation counter after the addition of ACS II.

Results

Cytotoxicity and antitumor activity of MS-247

The in vitro cytotoxic activity of MS-247 was evaluated in four murine tumor cell lines (L1210 leukemia, P388 leukemia, Colon 26 carcinoma and B16 melanoma; Table 1). The IC_{50} values of MS-247 against these cell lines ranged from 11 n*M* (L1210) to 500 n*M* (B16). The in vivo antitumor activities of MS-247 were compared with those of several clinically approved anticancer agents. MS-247 displayed antitumor activity following a single i.v. administration against three of the four tumor cell lines examined. In particular, MS-247 exhibited marked activity against L1210 tumors implanted in mice. Half of the mice receiving 21 and 30 mg/kg survived for the entire period of the experiment (Table 2), suggesting that just one i.v. injection of MS-247 killed all the L1210 cells in ascites in half of the mice. On the other hand, no cured mice were observed in groups treated with ADM, CDDP or paclitaxel at their MTDs (ADM approximately 30 mg/kg, CDDP 15 mg/kg, paclitaxel 30 mg × 5) as judged from their shortened lifespans compared with the control group.

MS-247 also showed apparent activity comparable to those of CDDP and paclitaxel against P388 leukemia while ADM at 21 and 30 mg/kg was markedly effective (four cured mice out of eight) (Table 3). MS-247 was also effective against solid tumors. At 10, 15 and 21 mg/kg, MS-247 showed strong activity against Colon26 tumors (Table 4). Comparing the tumor volumes at the MTDs of the four antitumor agents, the activity of MS-247 (T/C 1% at 21 mg/kg) was clearly greater than those of the other agents (ADM T/C 12% at 21 mg/kg,

Table 1 Growth-inhibitory activity of MS-247 against murine tumor cell lines in terms of its IC_{50} value. Cells were cultured for 3 days with MS-247. Cell growth was determined by an MTT assay

	L1210	P388	Colon26	B16
IC_{50} (n*M*)	11	73	280	500

Table 2 Antitumor activity of MS-247 administered i.v. against i.p.-inoculated murine L1210 leukemia cells. Cells were inoculated on day 0. Drugs were administered on day 1 (MS-247, ADM and CDDP) or days 1–5 (paclitaxel). Survival was monitored until day 60 (*ILS* increase in lifespan)

	Dose (mg/kg)	Lifespan (days)		ILS (%)	Survivors/ total
		Median	Range		
Control		8	7–10	0	0/8
MS-247	4.8	11	10–12	38*	0/8
	10	14	13–15	75*	0/8
	15	15	14–60	88*	1/8
	21	43	16–60	> 438*	4/8
	30	60	11–60	> 650*	5/8
	43	6	6–8	−25	0/8
ADM	10	11	10–12	38*	0/8
	15	13	11–47	63*	0/8
	21	15	14–18	88*	0/8
	30	17.5	9–24	119*	0/8
CDDP	10	12	10–15	50*	0/8
	15	13.5	13–16	69*	0/8
	21	14	6–17	75	0/8
Paclitaxel	30 × 5	9	9–11	13	0/8
	43 × 5	10	5–10	25	0/8

* $P < 0.001$ (Steel test)

CDDP T/C 5% at 15 mg/kg and paclitaxel T/C 33% at 43 mg/kg × 5). However, in B16 melanoma, MS-247 did not confer a benefit because one mouse died from toxicity at 21 mg/kg (T/C calculated as 17%) and the MTD (15 mg/kg) showed no antitumor effect (T/C 96%), while apparent antitumor effects were observed at the MTDs of the other agents (ADM T/C 19% at 21 mg/kg, CDDP T/C 17% at 21 mg/kg and paclitaxel T/C 10% at 30 mg/kg × 5; Table 5).

Quenching of Hoechst 33342 DNA fluorescence by MS-247

MS-247 has a DNA minor groove-binding moiety that is based on netropsin. We have already studied the interaction of MS-247 with the DNA minor groove by a displacement assay using a well-known DNA minor groove-binder, Hoechst 33342 [25]. Fluorescence of the Hoechst 33342-DNA complex was decreased following the addition of MS-247 in a dose-dependent manner in both cell-free and cellular systems (Fig. 2), indicating that MS-247 competes with Hoechst 33342 for interaction in the same place of the DNA minor groove as already described [25]. Interestingly, the addition of Hoechst 33342 10 min after mixing MS-247 with DNA induced fluorescence at a very similar intensity to that induced when MS-247 was added to a mixture of Hoechst 33342 and DNA (Fig. 2a), suggesting that the interaction between MS-247 and DNA was as reversible as that of Hoechst 33342 and DNA over a short period (10–20 min).

Table 3 Antitumor activity of MS-247 administered i.v. against i.p.-inoculated murine P388 leukemia cells. Cells were inoculated on day 0. Drugs were administered on day 1 (MS-247, ADM and CDDP) or days 1–5 (paclitaxel). Survival was monitored until day 60 (*ILS* increase in lifespan)

	Dose (mg/kg)	Lifespan (days)		ILS (%)	Survivors/ total
		Median	Range		
Control		10	10–12	0	0/8
MS-247	10	14.5	13–16	45*	0/8
	15	14.5	13–16	45*	0/8
	21	15	13–17	50*	0/8
	30	17	16–19	70*	0/8
	43	8	5–9	−20*	0/8
ADM	4.8	13.5	12–15	35*	0/8
	10	17	16–20	70*	0/8
	15	21	19–29	110*	0/8
	21	43	21–60	> 330*	4/8
	30	59	23–60	> 490*	4/8
CDDP	2.3	12.5	12–18	25*	0/8
	4.8	14	13–14	40*	0/8
	10	16.5	16–18	65*	0/8
	15	19	18–23	90*	0/8
Paclitaxel	21 × 5	12.5	11–18	25	0/8
	30 × 5	13	12–14	30*	0/8
	43 × 5	13	12–16	30*	0/8
	62 × 5	16	12–19	60*	0/8

* $P < 0.001$ (Steel test)

Table 4 Antitumor activity of MS-247 administered i.v. against murine Colon26 carcinoma. Cells were inoculated s.c. on day 0. Drugs were administered on day 1 (MS-247, ADM and CDDP) or days 1–5 (paclitaxel). Tumor weights were measured on day 15. T/C (treatment/control) was calculated from the survivors in groups with dead mice

	Dose (mg/kg)	Tumor weight (g) (mean ± SD)	T/C (%)	Body weight change day 8 (g)	Toxic deaths[a]
Control		1.12 ± 0.32	100	0.4	0/8
MS-247	4.8	0.76 ± 0.23	68	−0.3	0/8
	10	0.21 ± 0.15	19*	−1.3	0/8
	15	0.09 ± 0.08	8*	−1.7	0/8
	21	0.01 ± 0.00	1*	−3.4	0/8
	30	0.00 ± 0.00	0*	−6.3	2/8
	43	–	–	–	8/8
ADM	4.8	0.99 ± 0.30	88	0.2	0/8
	10	0.50 ± 0.28	44*	0.0	0/8
	14	0.49 ± 0.22	44*	0.2	0/8
	21	0.13 ± 0.14	12*	−1.4	0/8
	30	0.04 ± 0.04	4*	−6.0	2/8
CDDP	4.8	0.79 ± 0.25	70	0.3	0/8
	10	0.18 ± 0.22	16*	−0.1	0/8
	15	0.05 ± 0.07	5*	−4.6	0/8
	21	0.02 ± 0.04	2	−6.0	5/8
	30	–	–	–	8/8
Paclitaxel	10 × 5	0.64 ± 0.34	57	−0.5	0/8
	21 × 5	0.57 ± 0.21	51*	−2.4	0/8
	30 × 5	0.62 ± 0.36	55	−3.2	0/8
	43 × 5	0.37 ± 0.20	33*	−4.2	0/8
	62 × 5	0.32 ± 0.17	29*	−5.7	1/8

*$P < 0.01$ (Steel test)
[a] Number of mice that had died by day 15

In the cell-free assay system, compound 3 (an inert form of MS-247; Fig. 1) showed the quenching activity while chlorambucil showed no quenching, indicating that the quenching activity of MS-247 is due to the displacement of Hoechst 33342 from the DNA minor groove by specific interaction of the DNA binding moiety of MS-247 and not due to ICL formation by the phenyl mustard residue as described bellow. In addition, compound 3 showed the quenching activity in the cell-free assay system (Fig. 2a) but not in the cellular system (Fig. 2b), suggesting that compound 3 was not able to penetrate into the cells.

Formation of DNA ICL by MS-247

Since MS-247 is considered to show ICL formation activity because of its phenyl mustard residue [10, 19], an alkaline elution assay was performed to detect ICL formation in the cells treated with MS-247. Exposure to

Table 5 Antitumor activity of MS-247 administered i.v. against murine B16 melanoma. Cells were inoculated s.c. on day 0. Drugs were administered on day 1 (MS-247, ADM and CDDP) or days 1–5 (paclitaxel). Tumor weights were measured on day 15. T/C (treatment/control) was calculated from the survivors in groups with dead mice

	Dose (mg/kg)	Tumor weight (g) (mean ± SD)	T/C (%)	Body weight Change day 8 (g)	Toxic deaths[a]
Control		0.60 ± 0.39	100	0.3	0/8
MS-247	15	0.57 ± 0.32	96	−1.3	0/8
	21	0.42 ± 0.21	17	−3.6	1/8
	30	0.17 ± 0.15	28	−4.7	5/8
	43	–	–	–	8/8
ADM	10	0.40 ± 0.29	67	0.3	0/8
	14	0.15 ± 0.09	26*	0.1	0/8
	21	0.11 ± 0.08	19*	−0.4	0/8
	30	0.07 ± 0.10	12*	−3.9	1/8
CDDP	10	0.50 ± 0.27	83	0.2	0/8
	15	0.38 ± 0.25	64	−1.6	0/8
	21	0.10 ± 0.15	17*	−4.5	0/8
	30	–	–	–	8/8
Paclitaxel	10 × 5	0.30 ± 0.27	50	−0.1	0/8
	21 × 5	0.20 ± 0.17	33	−0.4	0/8
	30 × 5	0.06 ± 0.04	10*	−3.1	0/8
	43 × 5	0.07 ± 0.05	12*	−4.4	1/8
	62 × 5	–	–	–	8/8

*$P < 0.01$ (Steel test)
[a] Number of mice that had died by day 15

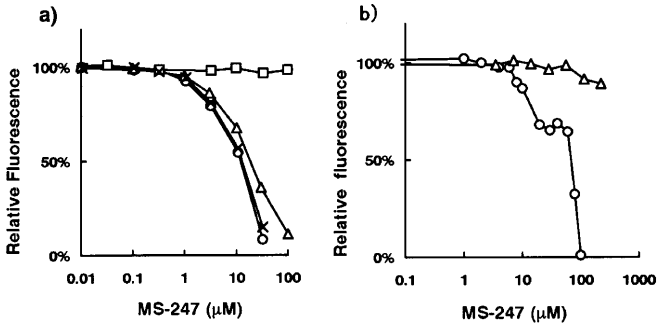

Fig. 2a,b Effects of MS-247 on Hoechst 33342 fluorescence. **a** Calf thymus DNA was preincubated with 6.1 μ*M* Hoechst 33342 for 10 min, followed by a further incubation for 10 min with MS-247 (○), chlorambucil (□) or compound 3 (△); otherwise DNA was preincubated with MS-247 for 10 min and, after that, 6.1 μ*M* Hoechst 33342 was added followed by incubation for 10 min (×). **b** L1210 cells were pretreated with 6.1 μ*M* Hoechst 33342 for 20 min, followed by MS-247 (○) or compound 3 (△) for a further 20 min

MS-247 for 6 h caused an apparent elution delay of ^{14}C-labeled DNA while exposure for 2 h did not affect the elution pattern in ^{14}C-labeled and UV-irradiated L1210 cells (Fig. 3). This result in relation to the incubation period suggests that ICL formation occurred relatively slowly compared to the reversible association of MS-247 with the DNA minor groove, which was detected within a few minutes as mentioned above.

ICL formation was directly confirmed using the plasmid DNA pBR322 in vitro. When DNA was treated with MS-247 at 20 μ*M* for 1–2 h, a new band twice the original size appeared on alkaline agarose gel as well as the crosslinker CDDP although treatment for 15 min did not cause a clear change (Fig. 4a). This result suggest that MS-247 directly induced ICL and the reaction occurred more slowly than the reversible association with the DNA minor groove in even naked DNA as mentioned above. Compound 3 showed no ICL formation at 1 μ*M* or 10 μ*M* while chlorambucil was able to form ICL at 10 μ*M* but not at 1 μ*M* (Fig. 4b). On the other hand, MS-247, which was made by chemical conjugation of the chlorambucil moiety with the DNA-binding moiety, showed apparent activity at 1 μ*M* and potent activity at 10 μ*M* (Fig. 4b), suggesting that chemical conjugation of these two moieties enabled the drug to target and bind DNA efficiently. The speed of ICL formation in the cell-free system seemed to be higher than that in the cellular system because ICL formation was detected within 2 h in the cell-free assay (Fig. 4a) while 6 h was needed in the cellular assay (Fig. 3). Therefore, it is possible that intracellular conditions such as high concentrations of basic proteins including histones and repairing systems for DNA damage might disturb and slow down the alkylation reaction.

Inhibition of macromolecule synthesis by MS-247

Cellular DNA can be said to be the primary target of the cytotoxic effect of MS-247 from the above data and from those of a previous study [25]. Therefore, we next compared the susceptibility of macromolecule synthesis (DNA, RNA and protein) to MS-247, by incorporation of [^{14}C]thymidine, [^{3}H]uridine and [^{3}H]leucine in L1210

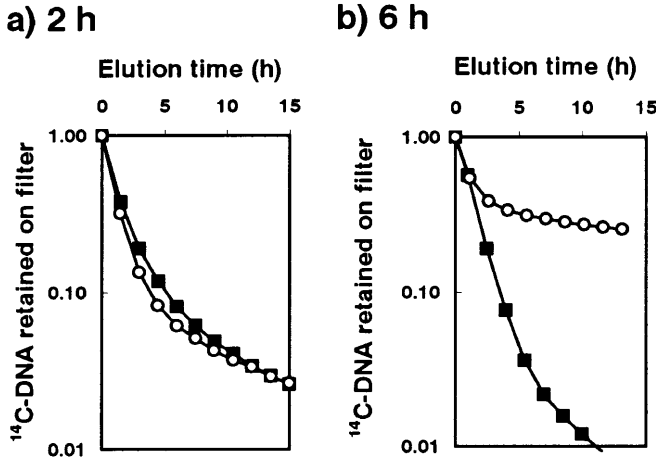

Fig. 3a,b DNA-DNA interstrand crosslinking in vivo. L1210 cells were labeled with [^{14}C]thymidine for 5 h and incubated further in the presence (○) or the absence (■) of 20 μ*M* MS-247 for 2 h (**a**) or 6 h (**b**) at 37 °C. After the cells were collected on the filter and lysed with a solution containing SDS and proteinase K, the filter was irradiated with UV and DNA was eluted with alkaline solution (pH 12.1)

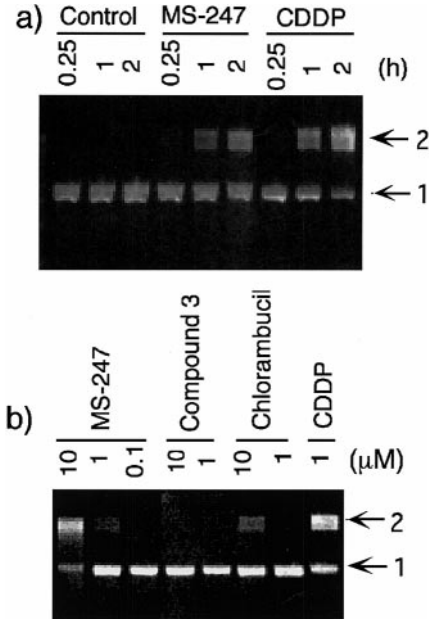

Fig. 4a,b ICL formation in plasmid DNA with MS-247. ICL formation with MS-247 was compared with ICL formation with CDDP (**a**), and the counterparts, compound 3 and chlorambucil (**b**). Plasmid DNA was incubated with MS-247 at 20 μ*M* or CDDP at 20 μ*M* for 0.25 h, 1 h and 2 h (**a**), or incubated with the agents at 0.1 μ*M*, 1 μ*M* and 10 μ*M* for 2 h (**b**), and the reaction mixture was electrophoresed on alkaline agarose gel

cells. Incorporation of [^{14}C]thymidine was slightly inhibited by incubation with 20 μ*M* MS-247 for 30 min and 2 h (Fig. 5a,b), and was strongly inhibited by incubation for 6 h (Fig. 5c). ICL formation could be detected following incubation with 20 μ*M* MS-247 for 6 h, but not following a 2-h incubation, as shown in the alkaline elution assay (Fig. 3), whereas the interaction with the DNA minor groove clearly occurred within 10–20 min as in the Hoechst 33342 quenching assay in the cellular system (Fig. 2). These results suggest that the ICL formation, but not the interaction with the DNA minor groove, mainly contributed to the inhibition of DNA synthesis and cell proliferation. In addition, the synthesis of RNA and protein was inhibited by longer incubation times and higher concentrations compared to DNA synthesis, suggesting that the synthesis of RNA and protein was inhibited after the impairment of DNA function by ICL formation.

Discussion

In the present study, we tested the in vitro cytotoxicity and in vivo antitumor activity of MS-247 in comparison with the clinically approved antitumor agents, ADM, CDDP and paclitaxel, against murine tumor cell lines which are popular for the evaluation of antitumor agents. MS-247 displayed various in vitro cytotoxicities, with IC_{50} values ranging 11 n*M* (L1210) to 500 n*M* (B16). The reported IC_{50} of tallimustine (30 ng/ml) [8] which structurally resembles MS-247 [23] against L1210 cells was equivalent to that of MS-247 while those of CC-1065 analogs, another class of DNA minor groove-binders analogous to MS-247, were 1000-fold higher than that of MS-247 [24]. MS-247 injected i.v. exhibited superior in vivo antitumor activity against i.p. L1210 cells (ILS 650%) in comparison with other antitumor agents that possess DNA minor groove-binding activity such as tallimustine (ILS 75%, i.p. dosing), bizelesin (ILS 78%), KW-2189 (ILS 44%) and adozelesin (ILS 100%) [6, 11, 13, 18]. Furthermore, MS-247 exhibited a superior activity also in Colon26 solid tumors (T/C = 1%) as compared to the DNA-binding agent, KW-2189 (T/C = 19%) [11]. However, in the present study, MS-247 did not show a clear antitumor benefit in B16 melanoma-bearing mice because of mortality at a moderate dose at which a reduction in tumor volume was seen in the other surviving mice. A single injection of MS-247 has been reported to show significant antitumor activity against a broad range of human tumor xenografts including those insensitive to paclitaxel and CPT-11 [25]. This observation and the findings of the present study suggest that MS-247 has potent antitumor activity against various types of tumor cells.

Yamori et al. [25] have previously reported that MS-247 interacts with the DNA minor groove, inhibits topoisomerases, blocks the cell cycle at the G_2/M phase, and finally induces apoptosis in a similarly manner to many other DNA minor groove-targeting drugs [4, 7, 23]. However, it has not been clarified how the chlorambucil moiety and the minor groove-binding moiety of the MS-247 molecule contribute to the impairment in DNA function and the exertion of cytotoxicity. Therefore, in the present study, we demonstrated the importance of the chlorambucil moiety and the DNA minor groove-binding moiety in ICL formation and the cytotoxicity of MS-247 using both cell-free and cellular assay systems. To ascertain whether MS-247 associates with the DNA minor groove, we used the fluorochrome, Hoechst 33342, which is known to reversibly bind to the minor groove in AT-rich regions of DNA, as a probe [14, 17, 22]. Fluorescence quenching of the Hoechst 33342-DNA complex by MS-247 occurred within a short period in a dose-dependent manner in both cell-free and cellular systems. In addition, the fluorescence intensity was the same if the MS-247 was added to the mixture of Hoechst 33342 and DNA or Hoechst 33342 was added to the mixture of MS-247 and DNA. This result suggests that, during the initial phase of the interaction with DNA, MS-247 is able to reversibly

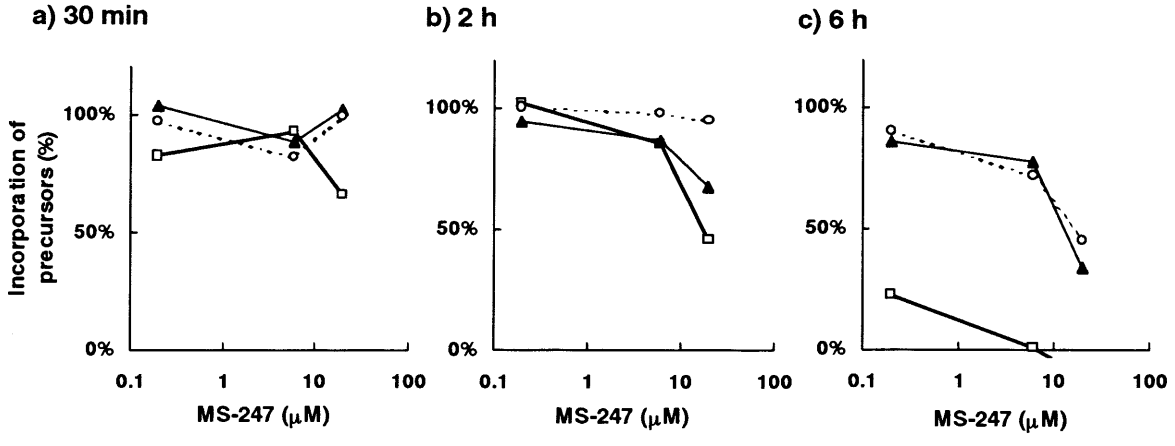

Fig. 5a–c Effects of MS-247 on the incorporation of labeled precursors into DNA (□), RNA (▲) and proteins (○) in L1210 cells. Cells were pretreated with MS-247 for 10 min (**a**), 100 min (**b**) and 340 min (**c**), and incubated for a further 20 min with the precursors [^{14}C]thymidine, [^{3}H]uridine or [^{3}H]leucine

occupy, but not bind covalently to, a certain region of the DNA at least partly identical to that occupied by Hoechst 33342 displacing Hoechst 33342 from that place.

We also confirmed the ICL formation by MS-247 in both cell-free and cellular systems using a DNA band-shift assay and an alkaline elution assay. In both assays, the incubation time needed for ICL formation (1 h or 6 h) was longer than that needed for displacement of Hoechst 33342 (10–20 min). Furthermore, chemical conjugation of the DNA minor groove-binding moiety to the alkylating moiety chlorambucil apparently potentiated the activity for ICL formation in comparison with chlorambucil alone. These findings including the result of quenching assay suggest that, at the beginning of the interaction, MS-247 binds to the DNA minor groove via the DNA-binding moiety and then gradually forms ICL at the binding sites via the chlorambucil moiety, and this mode of action might enable MS-247 to target and crosslink DNA efficiently.

Finally, we studied the influence of MS-247 on macromolecule synthesis in L1210 cells. Exposure of the cells to MS-247 at 0.2 μM or 6 μM for up to 2 h showed little effect on macromolecule synthesis, but continuous exposure for 6 h to the same concentration of MS-247 significantly or strongly inhibited DNA synthesis. Based on these results and the observation that a 6-h incubation period was needed for ICL formation in the cells, the inhibition of DNA synthesis might be mainly due to ICL formation, but not to the reversible occupation of the DNA minor groove. The structurally similar compound tallimustine has been reported to produce no ICL formation and no significant changes in DNA synthesis following treatment for up to 12 h [5, 9]. Therefore, the mode of action of MS-247 may be different from that of tallimustine, although we do not have conclusive evidence from a direct comparison in the same experiment.

In this study, we demonstrated that MS-247 binds DNA, forms ICL and inhibits DNA synthesis. Furthermore, ICL formation was demonstrated to be one of the major mechanisms of action of this agent. MS-247 showed potent antitumor activity comparable or superior to that of paclitaxel, CDDP and ADM against murine tumors and exhibited a strong activity against a broad range of human xenografts. Therefore, we consider that MS-247 may be a useful agent for treatment of clinical cancers.

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