COMPARATIVE ANTITUMOR ACTIVITIES OF 7-*N*-(*p*-HYDROXYPHENYL)MITOMYCIN C (M-83) AND MITOMYCIN C

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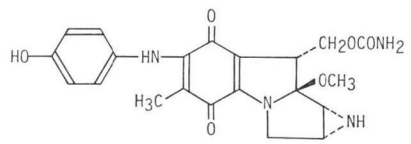
(Received for publication December 27, 1982)

The antitumor activity of 7-*N*-(*p*-hydroxyphenyl)mitomycin C (M-83) against 7 kinds of ascitic tumors and 4 kinds of solid tumors was compared with that of mitomycin C (MMC). M-83 showed more potent activities than MMC against ascites sarcoma 180, fibrosarcoma Meth 1, sarcoma Meth A, melanoma B-16, leukemia P388 and lymphoma EL4, by a single intraperitoneal injection. Furthermore, M-83 gave markedly higher chemotherapeutic ratio than MMC in these tumor systems.

M-83 was also markedly effective against solid tumors of sarcoma 180, Meth 1, Meth A and Lewis lung carcinoma, by a single intravenous injection. M-83 gave lower myelo-suppression than MMC at the doses which gave almost equal inhibition on the tumor growth of solid Meth 1. M-83 and MMC significantly inhibited the growth of HeLa S_3 cells. Cell growth was observed at 24 hours after addition of 3×10^{-3} mM of drugs, but no growth was shown thereafter. M-83 inhibited more strongly the incorporation of the radioactive precursor into DNA than that into RNA or protein at the concentration of 3×10^{-3} mM.

We have already reported that 7-*N*-(*p*-hydroxyphenyl)mitomycin C (M-83) was the most effective against P388 leukemia among 7-*N*-phenyl derivatives of mitomycin C (MMC) which had been shown to give higher activity than MMC in USUBUCHI[2] carcinoma. M-83 gave higher antitumor activity and chemotherapeutic ratio than MMC against leukemia P388[3] and a 3-methylcholanthrene induced fibrosarcoma (Meth 1) in an i.p.-i.p. system. In addition to its superior activity, M-83 was less myelosuppressive than MMC on the peripheral white blood cell count and the number of bone marrow cells at the doses which gave equivalent inhibition of tumor growth on sarcoma 180.[4] In this paper the antitumor activity of M-83 and MMC against various experimental murine tumor models including i.p.-i.p., i.p.-i.v. and s.c.-i.v. system is compared in detail. The growth inhibitory activity and the effect on macromolecule synthesis of M-83 was also shown in HeLa S_3 cells.

Fig. 1. Structure of M-83.



7-*N*-(*p*-Hydroxyphenyl)mitomycin C
(NSC 278891) (M-83)

Materials and Methods

Animals

Male mice of *dd*Y, CDF_1 (BALB/c×DBA/2) and BDF_1 (C57BL/6×DBA/2) strains were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Hamamatsu). The mice were 6~8 weeks old (19~28 g) at the time of tumor inoculation.

Tumors

Sarcoma 180 was supplied by the National Cancer Center, Research Institute (Tokyo) and has been

passaged weekly by intraperitoneal inoculation in *dd*Y mice. Lymphocytic leukemia P388, lymphoid leukemia L1210, melanoma B-16 and Lewis lung carcinoma were donated by the Cancer Chemotherapy Center, Tokyo, and lymphoma EL4 and sarcoma Meth A were donated by the Research Institute for Tuberculosis and Cancer, Tohoku University, Sendai, and Sasaki Institute, Tokyo, respectively. These tumors have been maintained by continuous passage in each syngeneic strain. Fibrosarcoma Meth 1, induced[5] in BALB/c mice by a cutaneous injection of 3-methylcholanthrane, has been maintained by weekly intraperitoneal passage in the same strain of mice. In experiments, sarcoma 180 (1×10^6), Meth 1 (1×10^6), Meth A (1×10^6), P388 (1×10^6), EL4 (1×10^6), L1210 (1×10^5) and B-16 (10% homogenate: 0.5 ml) were inoculated intraperitoneally into *dd*Y, CDF_1 and BDF_1 mice, respectively. Sarcoma 180 (5×10^6), Meth 1 (5×10^6) and Meth A (5×10^6) were inoculated subcutaneously at the axillary or abdominal region of *dd*Y or CDF_1 mice, respectively. Small fragments ($2\times2\times2$ mm) of Lewis lung carcinoma were inoculated by trocar subcutaneously into axillary region of BDF_1 mice. L1210/MMC was kindly provided by Dr. S. FUJIMOTO of the Cancer Chemotherapy Center.

Chemicals

Mitomycin C (MMC) and M-83, were obtained from Kyowa Hakko Kogyo Co., Ltd. MMC was dissolved in physiological saline. M-83 was dissolved in physiological saline containing Tween 80 (Wako Pure Chemical Ind.), as previously reported.[1] These compounds were administered once the day after the tumor inoculation.

Evaluation of Antitumor Activity

Antitumor activity of the test compounds against the ascitic form of 7 kinds of tumors used in this experiment was assessed as a percentage of the increase in life span of the treated group over untreated group (ILS%). The chemotherapeutic ratio (C. R.) was defined as the ratio of a dose providing maximum effectiveness (optimal dose) to a dose giving 30% of ILS (minimum effective dose: MED). Antitumor activity of the test compounds against solid forms used in this experiment was represented as the ratio of tumor volume of mice administered with test compounds to that of control mice. Tumor sizes were measured periodically with calipers. Tumor volume was calculated by the formula represented in the NCI Protocols for screening[6]: Volume (mm^3)$=1/2\ ab^2$, where a and b represent the larger and smaller diameter, respectively.

Culture of HeLa S_3 Cells

HeLa S_3 cells used in this study were kindly supplied by Dr. K. KURODA of the National Institute of Genetics (Mishima) and maintained in Eagle's minimum essential medium (Nissui Seiyaku Co., Ltd.) supplemented with 10% fetal bovine serum (Grand Island Biological Company; Gibco). The cells (3×10^4/ml) were plated into 24-wells Multidish (NUNC Co., Ltd.) and incubated in a 5% CO_2 and air at 37°C. The treatment of cells with the drugs was carried out at 24 hours after incubation. After incubation with drugs, the cells were washed twice with phosphate buffered saline (PBS, pH 7.2) and then fed with fresh medium for further incubation. The number of cells was counted by a standard hemocytometer. The precursor incorporation was measured by pulse-labeling of the cells with [^{3}H]thymidine (0.5 μCi/ml, 22.9 Ci/mM) (The Radiochemical Centre, Amersham), [^{3}H]uridine (0.5 μCi/ml, 30.0 Ci/mM) or [^{3}H]leucine (1.2 μCi/ml, 52.0 Ci/mM) for 60 minutes. Immediately after incubation at 37°C, the medium containing radioactive precursors was removed by aspiration, and the adherent cells were washed three times with cold PBS followed by successive washes with 5% trichloroacetic acid (TCA) for 30 minutes and then with ethanol for 10 minutes. The dried cell residues were dissolved in 1 ml of 0.5 N NH_4OH and the radioactivity of aliquot was counted by a liquid scintillation counter.

Acute Toxicity

M-83 and MMC were administered once intravenously into CDF_1 and BDF_1 mice. Each experimental group comprised 18 animals. The LD_{50} was calculated at 14th day after the injection of drug by the method of LITCHFIELD and WILCOXON.[7]

Measurement of Peripheral White Blood Cells

Twenty μl samples of supraorbital venous plexus blood of the mice were mixed with 9.98 ml of Cell kit-7 (Toa Medical Electro. Co., Ltd.) solution and counted by Microcell counter (Toa Medical Electro-

nics) after lyisis of erythrocytes with saponin solution.

Results

Acute Toxicities of M-83 in Mice

The LD_{50} values of M-83 by a single intravenous injection in CDF_1 and BDF_1 mice were 20.2 and 18.8 mg/kg, respectively, by LITCHFIELD-WILCOXON method and were about 2.5 to 3.0 times higher than that of MMC, as shown in Table 1. The LD_{50} value of both compounds was higher in BDF_1 mice than CDF_1 mice.

Antitumor Activities of M-83 against Ascitic Tumors

The comparative antitumor activities of M-83 and MMC against 7 kinds of ascitic tumors used in this experiment were listed in Table 2. M-83 showed higher maximum ILS% (ILS max%) than MMC against all ascitic tumors used in this study (except L1210), and long survivors were detected in the mice bearing sarcoma 180, Meth 1, Meth A, B-16, P388 and L1210 tumors treated with M-83. The minimum effective doses (MED) of both compounds were almost equal in B-16, P388, EL4 and L1210 and MEDs of M-83 for sarcoma 180, Meth 1, Meth A were smaller than those of MMC. The optimal dose of M-83 by a single intraperitoneal injection in these tumor systems were 10 to 20 mg/kg, and were roughly 2 to 5 times higher than MMC. Therefore, M-83 showed clearly higher chemotherapeutic ratio than MMC in these tumor systems.

Table 1. Acute toxicities of M-83 and MMC in normal mice.

Drugs	Strain	LD_{50} (mg/kg)	95% confidence limits (mg/kg)
M-83	CDF_1 mice	20.2	19.0~21.6
	BDF_1 mice	18.8	18.0~19.6
MMC	CDF_1 mice	8.2	7.7~ 8.7
	BDF_1 mice	6.3	6.0~ 6.7

Drugs were once injected intravenously.

Table 2. Comparison of antitumor efficacies of M-83 and MMC against murine ascitic tumors.

Tumor systems	Route	M-83				MMC			
		OD[a] (mg/kg)	MED[b] (mg/kg)	CR[c]	ILS max[c] (%)	OD[a] (mg/kg)	MED[b] (mg/kg)	CR[c]	ILS max[d] (%)
Sarcoma 180	i.p.	20	0.063	318	178(3/5)[e]	6	0.50	12	108(1/5)[e]
Fibrosarcoma Meth 1	i.p.	10	0.156	64	444(4/5)	6	0.80	8	300(1/5)
Sarcoma Meth A	i.p.	10	0.19	53	301(3/5)	6	0.58	10	212(1/5)
Melanoma B16	i.p.	20	0.21	95	215(3/5)	4	0.37	11	118
Leukemia P388	i.p.	20	0.31	65	286(2/5)	5	0.38	13	80
Leukemia P388	i.v.	20	11.5	2	56	6	—	—	21
Lymphoma EL4	i.p.	10	6.0	2	53	6	5.8	1	31
Leukemia L1210	i.p.	20	2.3	9	44	4	2.5	2	53
Leukemia L1210/MMC[f]	i.p.	10	1.25	9	107	6	—	—	21

Tumor cells were implanted intraperitoneally on day 0 and the drugs were injected intraperitoneally (i.p.) or intravenously (i.v.) into the mice on day 1.

a) Optimal dose shows the dose with ILSmax%.

b) Minimum effective dose shows the dose with 30% of ILS.

c) Chemotherapeutic ratio shows the ratio of optimal dose to minimum effective dose.

d) Maximum increased in life span.

e) The number in parenthesis are 60 or 90 days survivors.

f) The resistant strain of L1210 against MMC.

Table 3. Effect of administration schedule on the antitumor activity of M-83 and MMC.

Compounds	Dose schedule	Administration day	Optimal dose[a] (mg/kg/day)	ILS max[b] (%)	CR[c]
M-83	Single	(Day 1)	15	57	2.6
	Intermittent	(Day 1, 5, 9)	10	70	2.9
	Successive	(Day 1~10)	5	67	3.3
MMC	Single	(Day 1)	6	44	1.7
	Intermittent	(Day 1, 5, 9)	4	57	1.9
	Successive	(Day 1~10)	1	33	1.9

P388 leukemia cells (1×10^5) were inoculated intravenously into CDF_1 mice on day 0. The compounds were given intravenously by single, intermittent and successive administrations. The mean survival days of control mice: 10.8 ± 0.4 days.

a) Optimal dose shows the dose with ILS max.

b) Maximum % of increase in life span.

c) Chemotherapeutic ratio shows the ratio of optimal dose to minimum effective dose which gave 30% of ILS.

Fig. 2. Effects of M-83 and MMC on the survival time of mice bearing melanoma B-16 (i.p.-i.p.).

Melanoma B-16 cells (10% homogenate: 0.5 ml) were implanted intraperitoneally into BDF_1 mice on day 0 and the drugs were injected intraperitoneally on day 1. Mean survival time of control group was 17.4 ± 1.5 days. The numbers in parenthesis were number of 60 days survivors per 5 mice.

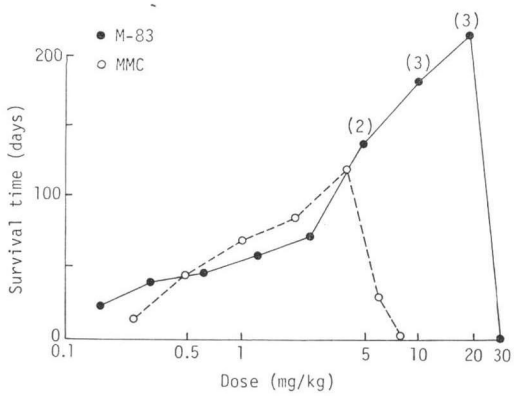


Table 4. Effects of M-83 and MMC on i.p.-i.v. system of P388 leukemia.

Compounds	Dose (mg/kg)	Survival days (mean±SD)	ILS (%)
Control	—	10.4 ± 0.9	—
M-83	25	5.6 ± 0.5	0
	20	16.2 ± 1.3	56
	15	14.8 ± 0.8	42
	10	12.8 ± 1.8	23
	5	11.6 ± 0.9	12
	2.5	11.0 ± 1.0	6
MMC	8	11.6 ± 0.5	12
	6	12.6 ± 1.8	21
	4	12.4 ± 2.5	19
	2	12.0 ± 1.6	15
	1	11.2 ± 0.8	8

P388 cells (1×10^6) were inoculated intraperitoneally into CDF_1 mice. Both compounds were injected once intravenously the day after inoculation.

M-83 also showed potent antitumor activity against the MMC resistant strain of L1210 leukemia, giving 107% of ILS. Fig. 2 shows the antitumor activity of M-83 and MMC against melanoma B-16 in detail. Both compounds showed almost equal activities at the same dose level under 4 mg/kg by a single injection. At the doses over 4 mg/kg, the therapeutic activity of MMC was diminished, but the ILS% of M-83-treated group significantly increased, giving 40 to 60% of 60 days survivors. ILS max% and chemotherapeutic ratio of M-83 were clearly higher than those of MMC in melanoma B-16.

The administration schedule did not significantly affect ILS max% and chemotherapeutic ratio of M-83 and MMC in i.v.-i.v. system of P388 leukemia (Table 3). M-83 gave prolongation of life span to mice bearing P388 leukemia transplanted intraperitoneally by a single intravenous administration (i.p.-i.v. system), but MMC was not effective in this i.p.-i.v. system of P388 leukemia (Table 4).

Antitumor Activities of M-83 against Solid Tumor

M-83 and MMC showed significant growth inhibition on solid tumor of sarcoma 180, Meth 1, Meth A and Lewis lung carcinoma by a single i.v. administration. Table 5 shows the optimal dose and T/C of each drug at optimal dose. M-83 was more effective than MMC against sarcoma 180, Meth 1 and Meth A, but less effective against Lewis lung carcinoma on the optimal dose. But from chemotherapeutic indices (CI; Ratio of LD_{50} to ED_{50}), M-83 was only superior to MMC on sarcoma 180 and Meth 1.

Table 6 shows the effect of M-83 and MMC on the tumor growth of Meth 1 fibrosarcoma and on the white blood cell counts of tumor bearing mice in detail. Both compounds were markedly active against Meth 1 tumor, and 15 mg/kg of M-83 represented 87% of inhibition on the tumor growth. An almost equal inhibition (T/C=0.22~0.27) on the tumor growth was observed in mice treated with 10 mg/kg of M-83 and 6 mg/kg of MMC. The M-83 was less suppressive on the white blood cell counts than MMC at the doses which gave the same extent of tumor growth inhibition. But myelo-suppression was almost comparable at the optimal dose of each drug (15 mg/kg for M-83; 6 mg/kg for MMC).

Effect of M-83 on the Growth of HeLa S_3 Cells

Fig. 3 shows the effect of M-83 and MMC on the growth of HeLa S_3 cells incubated for 30 minutes at the concentrations of 3×10^{-2}, 3×10^{-3} and 3×10^{-4} mM. The growth of HeLa S_3 cells was inhibited

Table 5. Comparison of antitumor activities of M-83 and MMC against solid tumors.

Tumor	Day of evaluation	M-83			MMC		
		OD[a] (mg/kg)	T/C	CI[b]	OD[a] (mg/kg)	T/C	CI[b]
Fibrosarcoma Meth 1	Day 7	15	0.13	4.8	6	0.27	2.9
Sarcoma 180	Day 7	15	0.28	4.0	6	0.47	2.0
Sarcoma Meth 1	Day 10	15	0.30	2.9	6	0.45	3.0
Lewis lung carcinoma	Day 15	15	0.28	2.6	6	0.12	2.5

Tumor cells were implanted subcutaneously on day 0 and the drugs were injected intravenously on day 1.

a) OD represents the optimal dose (mg/kg) which gave the highest T/C without dying mice.

b) CI shows the ratio of LD_{50} to ED_{50}.

Table 6. Effect of M-83 and MMC on Meth 1 fibrosarcoma (s.c.-i.v.).

Drugs	Dose (mg/kg)	Tumor volume (mm^3)		WBC count[a] (cells/mm^3)	Mortality (Day 7)
		Mean±SD	T/C		
Control	—	686±108	—	11350	0 / 6
M-83	20	22± 12	0.03	1460	1 / 5
	15	89± 61	0.13	2820	0 / 5
	10	149± 47	0.22	6060	0 / 5
	5	337± 92	0.49	6860	0 / 5
	2.5	435±115	0.63	9600	0 / 5
	1.25	591±216	0.86	11060	0 / 5
MMC	8	78± 34	0.11	1300	1 / 5
	6	184± 84	0.27	2250	0 / 5
	4	254±112	0.37	3340	0 / 5
	2	402±104	0.59	7640	0 / 5
	1	620±121	0.90	10880	0 / 5

Meth 1 cells (5×10^6) were implanted subcutaneously into CDF_1 mice on day 0 and the drugs were injected intravenously on day 1.

a) White blood cell counts on day 5.

Fig. 3. Effect of M-83 and MMC on the growth of HeLa S_3 cells.

Logarithmically growing HeLa S_3 cells (5×10^4) were incubated with MMC and M-83 in MEM supplemented with 10% fetal bovine serum, 1 mM L-glutamin and 0.5% $NaHCO_3$. Number of cells was counted every day.

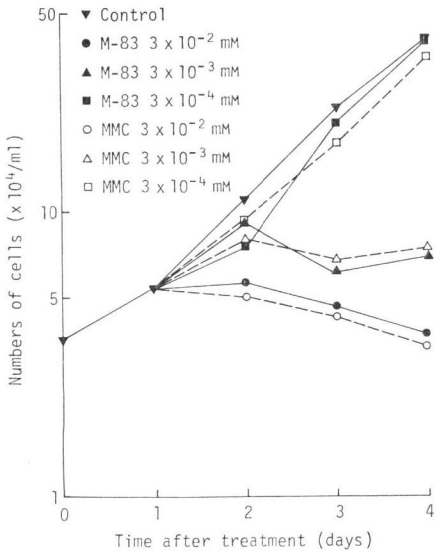
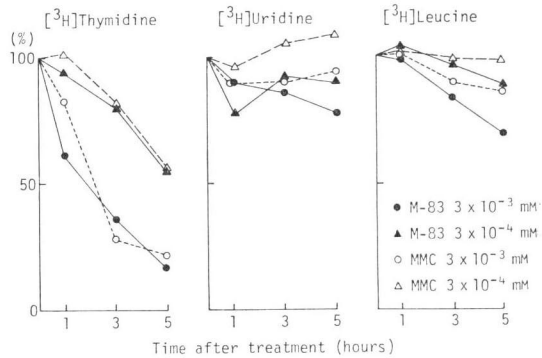


Fig. 4. Effect of M-83 and MMC on macromolecular synthesis of HeLa S_3 cells.

HeLa S_3 cells (3×10^4) were cultured for 2 days at 37°C in humidified 5% CO_2 in air. The labeled precursors were added at 0.5, 2.5 and 4.5 hours after addition of M-83 or MMC. Cells were incubated with radio-labeled precursors for 1 hour and the radioactivity of TCA insoluble fraction was measured.



by 3×10^{-2} and 3×10^{-3} mM of M-83 and the growth inhibitory effect of M-83 on HeLa S_3 cell was roughly equal to that of MMC at the molar equivalent concentration.

Effect of M-83 on the Macromolecule Synthesis

Both M-83 and MMC inhibited the incorporation of [^{3}H]thymidine into DNA more significantly than those of [^{3}H]uridine into RNA or [^{3}H]leucine into protein as shown in Fig. 4.

Discussion

The antitumor activities of M-83 against 7 kinds of ascitic tumors and 4 kinds of solid tumors were compared with those of MMC by a single intraperitoneal or intravenous injection. In the i.p.-i.p. system, M-83 showed more potent survival effects than MMC in leukemia P388, leukemia EL4, sarcoma 180, sarcoma Meth A, fibrosarcoma Meth 1, melanoma B16 and leukemia L1210/MMC. M-83 also gave a higher chemotherapeutic ratio in these tumor systems, because the optimal dose of M-83 was 2~5 times higher than that of MMC and minimum effective dose (MED) of M-83 was almost equal to that of MMC. But M-83 was only as effective as MMC for prolongation of life span of mice bearing L1210 leukemia transplanted intraperitoneally. The relative insensitivity of M-83 to i.p.-i.p. system of L1210 leukemia, as compared with another i.p.-i.p. systems, might result from higher metastasizing properties of L1210 cells than those of other tumor cells.[8] M-83 was also only equally effective to MMC on the i.v.-i.v. system of P388 leukemia which tumor cells could rapidly distribute in the whole body after injection of cells.[8]

From the pharmacokinetic study in mice, the transfer rate of M-83 from the blood to various tissues was shown to be lower than that of MMC.[9] The *in vitro* growth inhibitory effect of M-83 was higher than that of MMC. From these results M-83 could be explained to be more effective than MMC to tumor models in which drug could attack cells without suffering from rapid metabolism or complicated delivery system. As M-83 was effective to i.p.-i.v. system of P388 in which MMC was ineffective, transfer of M-83 to intraperitoneal cavity from blood may be much faster than that of MMC. M-83 may be

effective clinically to treat peritonitis carcinomatosa by i.v. injection.

As M-83 was effective on L1210/MMC, the effect on mitomycin resistant strains would be interesting for further studies to get the different antitumor spectrum from MMC. As shown in the previous reports, M-83 was confirmed to be less myelo-suppressive than MMC with doses which gave the same extent inhibition of tumor growth in mice bearing S-180 or Meth A (unpublished data).

The differences in the antitumor activity and bone marrow toxicity, as compared with MMC, suggested to us M-83, as one of the interesting derivatives of MMC for further clinical evaluation.

Acknowledgment

We are grateful to Dr. S. FUJIMOTO for giving us L1210/MMC cells.

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