

Effects of anthracycline derivatives on hepatic neoplastic nodules of Lewis lung carcinoma and colon adenocarcinoma 26

M. Iigo, K. Nishikata, Y. Nakajima & A. Hoshi

Chemotherapy Division, National Cancer Center Research Institute, Tsukiji 5-chome, Chuo-ku, Tokyo 104, Japan.

Summary Five anthracycline derivatives, i.e. doxorubicin, epirubicin, pirarubicin, aclarubicin and a new fluorinated anthracycline derivative (ME2303), were tested for antitumour activity in mice with hepatic neoplastic nodules of Lewis lung carcinoma and colon adenocarcinoma 26. Intravenous administrations of pirarubicin and ME2303 on day 4 or days 4, 8 and 12 in mice with hepatic neoplastic nodules of Lewis lung carcinoma rendered more than 50% of mice tumour-free over wide ranges of nontoxic doses, whereas a few mice were cured by treatment with doxorubicin and no mice were cured by treatment with epirubicin or aclarubicin. Moreover, when ME2303 was administered at 50 mg kg⁻¹ on days 7, 11 and 15 to six mice bearing more advanced hepatic tumours, five were cured, while pirarubicin and doxorubicin never achieved cure. Furthermore, in mice with hepatic neoplastic nodules of colon adenocarcinoma 26, ME2303 also showed a marked antitumour effect compared to pirarubicin or doxorubicin. Two or three injections of ME2303 starting from day 7 conferred a greater antitumour effect than did more fractionated or single-dose regimens.

Doxorubicin is widely used as a chemotherapeutic agent against various human neoplasms, and it shows impressive anti-tumour activities. However, its use is restricted due to its cardiotoxic and undesirable side effects. In order to improve the pharmacodynamic properties of clinically-useful anti-tumour anthracyclines, new doxorubicin analogues that have lower toxicity but higher antitumour activity have been synthesised or isolated (Oki *et al.*, 1975; Umezawa *et al.*, 1979; Cassinelli *et al.*, 1984; Weiss *et al.*, 1987; Arcamone, 1987; Israel *et al.*, 1987). In Japan, the presently-used anthracycline derivatives are doxorubicin, epirubicin, pirarubicin, aclarubicin and daunorubicin. Recently, Tsuchiya *et al.* (1986, 1988) reported ME2303 (Figure 1), a 2-fluoroglycoside of doxorubicin, which is more resistant to hydrolysis to aglycones and shows marked antitumour effects.

The liver is the primary site of metabolism of many drugs. The doxorubicin level in the liver is 100-fold higher than that in the plasma (Iguchi *et al.*, 1985). It would be interesting to know which anthracycline derivative is the most active against tumours in the liver. Moreover, ME2303 is quite resistant to hydrolysis to aglycones, and may be expected to be particularly effective against such tumours. Therefore, artificial hepatic 'metastases' (hepatic neoplastic nodules) were produced in mice by intrasplenic injection of Lewis lung carcinoma or colon adenocarcinoma 26 cells, and we then investigated the antitumour effects of various anthracycline derivatives.

Materials and methods

Chemicals

Doxorubicin and epirubicin were purchased from Kyowa Hakko Kogyo Co. Ltd, Tokyo, Japan. Pirarubicin and ME2303 were provided by Meiji Seika Kaisha Ltd, Tokyo. Aclarubicin was purchased from Sanraku-Ocean Co. Ltd, Tokyo.

Animals

Groups of six more specific pathogen-free male BDF₁ or CDF₁ mice weighing 22–24 g (Japan SLC, Inc.; Hamamatsu, Japan) were housed in plastic cages with wood chip bedding and were provided CA-1 pellet diets (CLEA Japan, Inc:

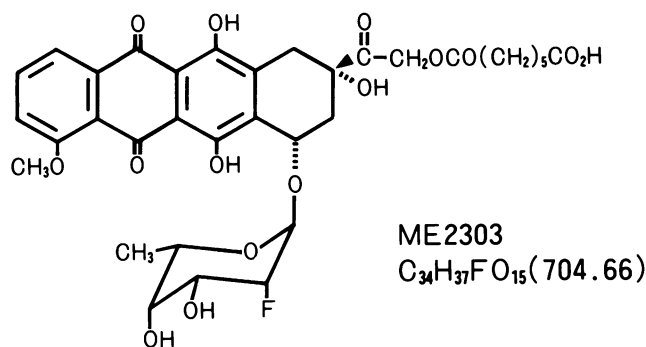


Figure 1 Structure of ME2303.

Tokyo, Japan) and water *ad libitum*. All experiments were performed in an animal laboratory at a controlled temperature (25°C).

Experimental procedure for inducing hepatic neoplastic nodules

Lewis lung carcinoma and colon adenocarcinoma 26 were maintained in male C57BL/6 and Balb/c mice, respectively. Cell suspensions of Lewis lung carcinoma and colon adenocarcinoma 26 in saline were prepared from surgically-removed corresponding tumours by disaggregating tumour pieces by gentle homogenisation in a loosely fitted glass homogeniser, and the cell suspension was passed through a 120-mesh sieve. Multiple hepatic neoplastic nodules were produced according to the method of Kopper *et al.* (1982). Mice were anaesthetised with ether, a left subcostal incision (about 5 mm) was made, and the spleen was externalised. A 27-gauge needle (Terumo Japan, Tokyo) was used to puncture the splenic capsule, and 5 × 10⁴ viable tumour cells in 0.1 ml of saline were injected directly into the upper pole of the spleen. Gentle pressure was applied for a period of 10 s to prevent hemorrhage and tumour cell extravasation. The arteria and vena linealis were then clamped with a medium hemoclip (Edward Weck & Co, Inc, NC), and the spleen was removed. The abdomen was stitched with surgical sutures, and the skin was closed with disposable skin clip applicators (Avlox 12, Medi Plast, Sweden). The mice were allowed to recover and were then randomised before being distributed to groups.

Drug treatment

Drugs were dissolved in 0.9% saline solution. The drugs were injected i.v. only on day 4, on days 4, 8 and 12 or on days 7,

11 and 15. Moreover, to determine the best treatment schedule of ME2303, the drug was injected i.v. using a 27 gauge needle (Terumo Japan, Tokyo) with gentle pressure in single (100 mg kg^{-1} , day 7) and fractionated-dose regimens ($50 \text{ mg kg}^{-1} \times 2$, $33 \text{ mg kg}^{-1} \times 3$, $20 \text{ mg kg}^{-1} \times 5$ and $11 \text{ mg kg}^{-1} \times 9$). The injection volume was 0.01 ml g^{-1} body weight. Observation was terminated on day 80. All mice surviving at that time were recorded as 'cured'. The antitumour effect was determined by comparing the mean survival time of each treated group with that of the control group and expressed as the increase in life-span (ILS). At death, the mice were examined for the presence of hepatic neoplastic nodules. Cured mice were excluded from the mean survival time calculation.

Statistical analysis

The *t*-test for small samples was used to determine the statistical significance of differences; $P \leq 0.05$ was considered significant.

Results

Antitumour effects of anthracycline derivatives on hepatic neoplastic nodules of Lewis lung carcinoma-bearing mice

Hepatic neoplastic nodules were created by intrasplenic inoculation of tumour cells. Under light microscopy, liver micronodules were clearly recognised on the 7th day after tumour cell inoculation of Lewis lung carcinoma and on the

4th day for colon adenocarcinoma 26. The liver weight at death was $3.24 \pm 0.11 \text{ g}$ ($n = 67$) or $5.42 \pm 0.26 \text{ g}$ ($n = 21$) in Lewis lung carcinoma or colon adenocarcinoma 26-bearing mice, whereas that of normal mice was $0.99 \pm 0.01 \text{ g}$ ($n = 60$). If untreated, all the mice died after approximately 20 days with many nodules in the liver.

The antitumour effects of five anthracycline derivatives (doxorubicin, epirubicin, pirarubicin, aclarubicin and ME2303) administered on day 4 or days 4, 8 and 12 were evaluated against established multiple murine hepatic nodules of Lewis lung carcinoma. The results of these anthracycline derivatives on this tumour system are shown in Table I. Two of six mice were cured by a single injection of doxorubicin on day 4 at 12.5 mg kg^{-1} , which was the best treatment protocol for doxorubicin. However, more than 50% of the mice were cured by pirarubicin at between 12.5 and 25 mg kg^{-1} in a single injection and 6.3 mg kg^{-1} t.i.d.

Moreover, more than 50% of the mice were cured by ME2303 at between 25 and 100 mg kg^{-1} in a single injection and between 25 and 50 mg kg^{-1} t.i.d., whereas no cured mice were recorded with epirubicin or aclarubicin. When therapy was initiated on day 7, ME2303 at 50 mg kg^{-1} t.i.d. was also effective, and higher rates of cure (5/6) were obtained, while doxorubicin and pirarubicin showed no cases of cure (Table II). The effect of pirarubicin initiated on day 7 was not as good as that obtained with treatment initiated on day 4. The effects of doxorubicin and pirarubicin decreased with the delay in the treatment. These observations clearly demonstrate the superiority of ME2303 over doxorubicin and pirarubicin in therapeutic efficacy against hepatic nodules of Lewis lung carcinoma.

Table I Effect of anthracycline derivatives on hepatic nodules of Lewis lung carcinoma

Drugs	Dose (mg kg^{-1})	Treatment schedule	MST ^a (days)	ILS (%)	No. of cured mice ^b
Doxorubicin	Control		21.4 ± 1.3^c		0/15
	25	Day 4	50.4 ± 2.9	136	0/6
	12.5		55.8 ± 7.5	161	2/6
	6.3		25.1 ± 2.0	17	0/7
	12.5	Days 4, 8, 12	18.0 ± 0.6	-16	0/6
	6.3		28.6 ± 2.8	34	1/6
	3.2		19.8 ± 0.8	-7	0/6
Epirubicin	Control		20.5 ± 1.6		0/6
	50	Day 4	10.7 ± 0.4	-48	0/6
	25		35.8 ± 2.0	75	0/6
	12.5		26.5 ± 0.6	29	0/6
	12.5	Days 4, 8, 12	35.7 ± 2.3	74	0/6
	6.3		26.8 ± 1.2	31	0/6
	3.2		23.3 ± 1.3	14	0/6
Pirarubicin	Control		20.2 ± 1.4		0/6
	25	Days 4			4/6
	12.5				5/6
	6.3		38.8 ± 3.1	92	2/6
	6.3	Days 4, 8, 12			6/6
	3.2		42.8 ± 5.8	112	1/6
	1.6		23.8 ± 1.3	18	0/6
Aclarubicin	Control		19.4 ± 1.5		0/6
	50	Day 4	8.5 ± 0.2	-56	0/6
	25		27.2 ± 1.4	40	0/6
	12.5		19.7 ± 1.3	2	0/6
	12.5	Days 4, 8, 12	24.2 ± 2.4	25	0/6
	6.3		25.0 ± 1.4	29	0/6
	3.2		22.2 ± 1.6	14	0/6
ME2303	Control		18.8 ± 1.7		0/10
	100	Day 4	37.3 ± 9.7	98	9/12
	75		50.0 ± 5.0	166	3/6
	50		40.7 ± 3.8	116	8/11
	25				4/6
	50	Days 4, 8, 12			6/6
	25				6/6
	12.5		37.3 ± 6.4	98	1/6
	6.3		27.2 ± 1.8	45	0/6

^aMST, mean survival time of deceased mice. ^bNumber of cured mice per treated mice, on day 80. ^cMean \pm s.e.

Table II Effect of anthracycline derivatives on advanced hepatic nodules of Lewis lung carcinoma

Drugs	Dose (mg kg ⁻¹)	MST ^a (days)	ILS (%)	No. of cured mice ^b
Control		17.6 ± 0.8 ^c		0/10
Doxorubicin	6.3	25.2 ± 2.2 ^d	43	0/6
	3.2	16.5 ± 1.0	-6	0/6
Pirarubicin	12.5	20.0 ± 0.8	14	0/6
	6.3	33.4 ± 1.3 ^e	90	0/6
	3.2	25.0 ± 2.0 ^e	42	0/6
ME2303	50			5/6
	25	39.8 ± 1.6 ^e	126	1/6
	12.5	29.3 ± 0.8 ^e	66	0/6

Lewis lung carcinoma cells were inoculated into the liver by intrasplenic inoculation, and drugs were administered i.v. on days 7, 11 and 15. ^aMST, mean survival time of deceased mice; ^bNumber of cured mice per treated mice, on day 80; ^cMean ± s.e.; ^d*P* < 0.01 compared to Control; ^e*P* ≤ 0.001.

Antitumour effect of anthracycline derivatives on hepatic neoplastic nodules of colon adenocarcinoma 26-bearing mice

For this study, we used colon adenocarcinoma 26 tumour cells. The hepatic neoplastic nodules showed a different histology from the nodules of Lewis lung carcinoma cells following intrasplenic inoculation. Micronodules of colon adenocarcinoma 26 were already found in the liver on day 4 (Figure 2). The antitumour effects of doxorubicin, pirarubicin

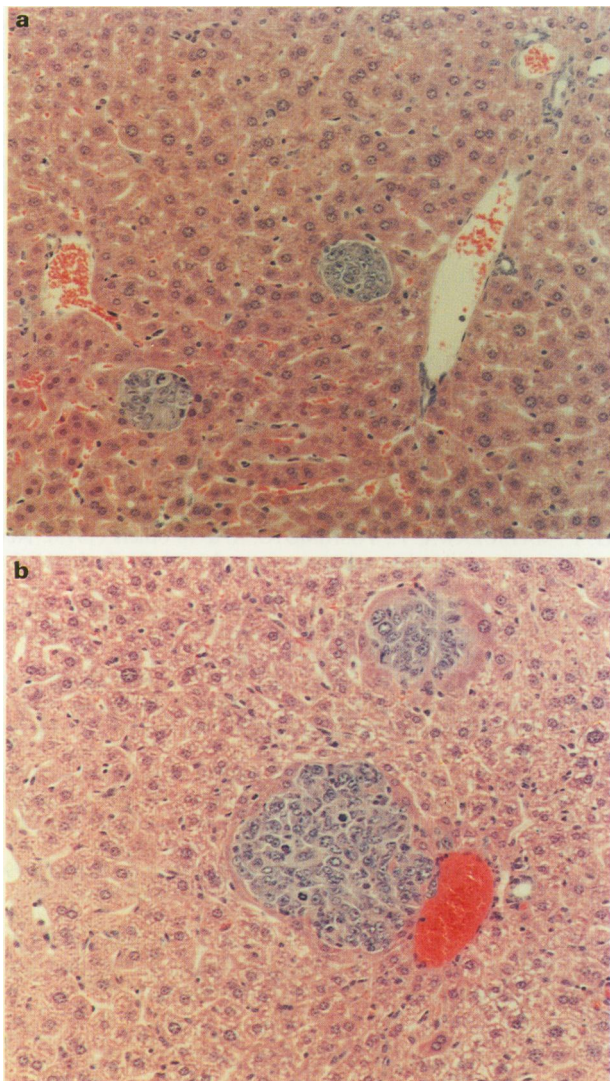


Figure 2 Liver tissue sections obtained from mice intrasplenicly injected with colon adenocarcinoma 26 on the 4th **a**, and 7th **b** days of tumour evolution. Haematoxylin and eosin × 170.

and ME2303 were examined in mice with intrasplenic inoculation of colon adenocarcinoma 26. There were few cases of complete tumour regression in these drug-treated mice on days 7, 11 and 15. The maximum ILSs of doxorubicin, pirarubicin and ME2303 were 58, 31 and 191%, respectively (Table III). ME2303 showed the strongest antitumour effect against this liver tumour.

Influence of treatment schedule of ME2303

To obtain more effective treatment with ME2303, the influence of the treatment schedule on the hepatic nodules of colon adenocarcinoma 26-bearing mice was investigated. With a total dose of 100 mg kg⁻¹ i.v., two- and three-fraction dose regimens showed greater efficacy compared to the single treatment regimen (Table IV). The five-fraction regimen (every other day) caused toxic death. The nine-fraction dose regimen (days 7–15 daily) also showed toxicity, but the toxicity of this regimen was weaker than that of the five-fraction dose regimen (days 7, 9, 11, 13 and 15). The body weight of the mice recovered after more than 2 weeks after stopping the treatment, but the hepatic neoplastic nodules regrew. Moreover, when compared with three- and five-fraction dose regimens in normal CDF₁ mice treated with same total doses of ME2303, the body weight with the five-fraction regimen was markedly decreased compared with the three-fraction regimen (Figure 3), like in tumour-bearing mice. This result means that the three-fraction dose regimen is lower in toxicity than the five-fraction regimen.

Discussion

In this study, we investigated the antitumour effect of five anthracycline derivatives (doxorubicin, epirubicin, pirarubicin, aclarubicin and ME2303) against hepatic nodules of Lewis lung carcinoma and colon adenocarcinoma 26. The hepatic nodules of Lewis lung carcinoma were well inhibited by pirarubicin and ME2303. These drugs produced many 'cured' mice when the drugs were injected on day 4 or on days 4, 8 and 12, while there were only a few cured mice in the group treated with doxorubicin using the same schedule.

Table III Effect of anthracycline derivatives on advanced hepatic nodules of colon adenocarcinoma 26

Drugs	Dose (mg kg ⁻¹)	MST ^a (days)	ILS (%)	No. of cured mice ^b
Control		16.0 ± 0.7 ^c		0/6
Doxorubicin	6.3	21.5 ± 2.6	34	1/7
	3.2	25.3 ± 3.9 ^d	58	0/7
Pirarubicin	12.5	20.0 ± 1.8	25	0/7
	6.3	21.0 ± 0.7 ^e	31	0/7
	3.2	17.6 ± 0.7	10	0/7
ME2303	50	46.6 ± 2.1 ^e	191	1/7
	25	28.1 ± 2.0 ^e	76	0/7
	12.5	21.7 ± 3.1 ^f	36	0/7

Colon adenocarcinoma 26 cells were inoculated into the liver by intrasplenic inoculation, and drugs were administered i.v. on days 7, 11 and 15. ^aMST, mean survival time of deceased mice; ^bNumber of cured mice per treated mice, on day 80; ^cMean ± s.e.; ^d*P* < 0.05 compared to Control; ^e*P* < 0.001; ^f*P* < 0.01.

Table IV Influence of treatment schedule of ME2303 on advanced hepatic nodules of colon adenocarcinoma 26

Dose (mg kg ⁻¹ day ⁻¹)	Treatment schedule	Mean survival time (days)	ILS (%)	Liver weight at death (g)
Control		19.6 ± 1.4 ^a		5.01 ± 0.47 ^a
100	Day 7	24.6 ± 1.0 ^b	26	4.66 ± 0.31
50	Days 7, 11	44.6 ± 6.1 ^c	128	4.23 ± 0.40
33	Days 7, 11, 15	49.3 ± 7.1 ^d	152	3.72 ± 0.49
20	Days 7, 9, 11, 13, 15	17.9 ± 0.5	-9	1.08 ± 0.29
11	Days 7–15, daily	29.1 ± 3.2 ^b	48	3.58 ± 0.55

^aMean ± s.e. of seven mice; ^b*P* < 0.05 compared to Control; ^c*P* < 0.01; ^d*P* = 0.001.

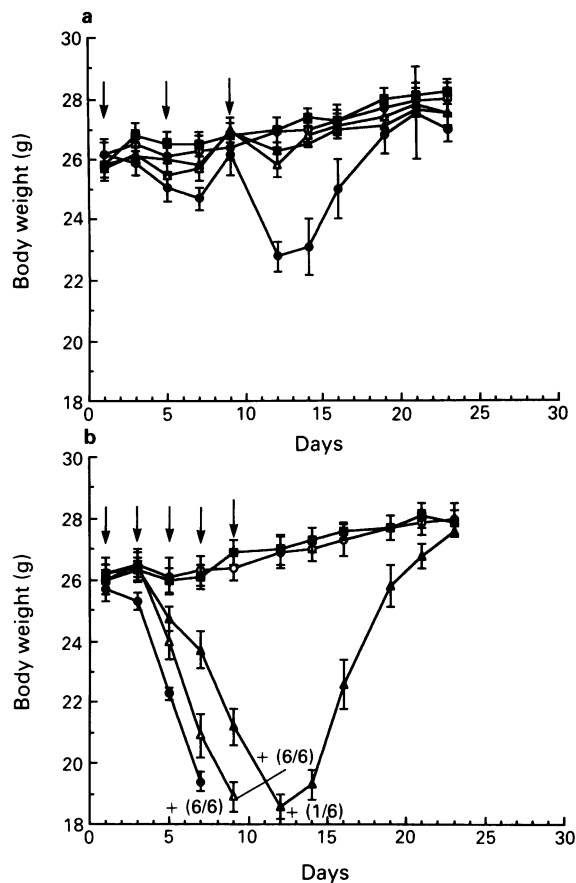


Figure 3 Effect on body weight of normal CDF1 mice in the three **a** and five- **b** fractionated dosage regimens of ME2303. Results are mean \pm s.e. of six mice + (dead mice/total number of mice). In **a**, \circ —, Control; \bullet —, $75 \text{ mg kg}^{-1} \text{ day}^{-1}$; \triangle —, $50 \text{ mg kg}^{-1} \text{ day}^{-1}$; \blacktriangle —, $30 \text{ mg kg}^{-1} \text{ day}^{-1}$; \blacksquare —, $15 \text{ mg kg}^{-1} \text{ day}^{-1}$. In **b**, \circ —, Control; \bullet —, $45 \text{ mg kg}^{-1} \text{ day}^{-1}$; \triangle —, $30 \text{ mg kg}^{-1} \text{ day}^{-1}$; \blacktriangle —, $18 \text{ mg kg}^{-1} \text{ day}^{-1}$; \blacksquare —, $9 \text{ mg kg}^{-1} \text{ day}^{-1}$.

On the other hand, no mice were cured by treatment with epirubicin or aclarubicin. Moreover, delayed treatment with

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