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Schedule-dependent and -independent Antitumor Activity of Paclitaxel-based Combination Chemotherapy against M-109 Murine Lung Carcinoma *in vivo*

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The established antitumor efficacy of paclitaxel against a variety of human tumors has led to preclinical and clinical studies to develop the paclitaxel-based combination regimens. We examined *in vivo* the antitumor activity and toxicity of the combination of paclitaxel and each of 8 antitumor agents, currently in clinical use, against M-109 murine lung carcinoma implanted subcutaneously into male CDF_1 mice. Paclitaxel given intravenously at 24 mg/kg/day on a schedule of consecutive daily injections for 5 days (d1–5) induced reproducibly, in 6 experiments, a significant (37–82%) increase in the survival time of tumor-bearing mice over saline-treated control mice. Cisplatin at 4 and 2 mg/kg/day given intravenously on the same treatment schedule showed no significant antitumor activity when given alone; however, the combination of paclitaxel at 24 mg/kg/day (d1–5) followed by cisplatin at a dose of 2 mg/kg/day (d6–10) induced a significant ($P<0.05$) prolongation of the survival time of tumor-bearing mice compared with the group given paclitaxel alone. On the other hand, treatment with these drugs on the reverse sequence caused toxic deaths of all mice. Such sequence-dependent toxic death of mice was also observed with the combination of paclitaxel and carboplatin, etoposide or methotrexate. The combination of paclitaxel and adriamycin, cyclophosphamide, ranimustine or vinblastine (VLB) showed a sequence-independent antitumor activity and a more-than-additive therapeutic effect was observed with the combination of paclitaxel and either VLB or ranimustine. Although the drug administration schedules used here may not be directly applicable to the clinic, knowledge of the nature of the sequence-dependency in paclitaxel-based combination chemotherapy should be useful in the design of clinical trials.

Key words: Paclitaxel — Combination chemotherapy — Schedule dependency — M-109 murine lung carcinoma — *In vivo*

Paclitaxel (Taxol) is a novel anticancer agent with activity against a variety of human tumors, particularly drug-refractory ovarian cancer,[1)] breast cancer[2)] and non-small-cell lung cancer.[3)] Paclitaxel has a characteristic ability to bind directly to β-tubulin *in vitro*[4)] and acts to stabilize polymerized tubulin into nonfunctional microtubule bundles,[5)] in contrast to vinca alkaloids that induce microtubule disassembly.[6)] Consequently, tumor cells are blocked in the late G_2 or mitotic phase of the cell cycle and are unable to replicate.[7)]

As with other chemotherapeutic agents, the clinical utility of paclitaxel will depend upon its optimum use in combination with other clinically useful agents. Ideally, agents used in such combinations should exhibit minimal overlapping toxicities and have confirmed effects that reflect at least additive, or preferably more-than-additive interactions in the target tumor cells. Therefore, in the present study, we examined the antitumor activity of the combination of paclitaxel and each of 8 antitumor agents currently in clinical use, i.e., cisplatin (CDDP), carboplatin (JM-8), etoposide (VP-16), methotrexate (MTX), adriamycin (ADM), cyclophosphamide (CPM), ranimustine (MCNU) and vinblastine (VLB), against M-109 murine lung carcinoma implanted subcutaneously. Another aim was to determine whether schedule-dependent antitumor activity and toxicity would be observed in paclitaxel-based combination chemotherapy, since previous studies had demonstrated sequence-dependent antitumor activity in the combination therapy of paclitaxel with cisplatin[8)] or vinorelbine tartrate.[9)]

We selected the M-109 murine lung carcinoma model for the following reasons: paclitaxel and some representative anticancer agents currently in clinical use (CDDP, ADM, CPM and VP-16) were modestly active against this tumor implanted subcutaneously, though no drug alone produced long-term survivors (cures) at a nontoxic dose.[10)] Therefore, any interaction between paclitaxel and those anticancer agents that resulted in extended survival dura-

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tion and/or cures in the absence of increased toxicity should be easily recognized as enhanced antitumor activity against this tumor.

MATERIALS AND METHODS

Animals and tumor The present study was approved by the Chiba Cancer Center Animal Care and Use Committee, in accordance with the Chiba Cancer Center guidelines for the care of laboratory animals. Adult male CDF_1 mice weighing 24–26 g were used in these studies. M-109 murine lung carcinoma was maintained subcutaneously in syngeneic adult female BALB/c mice. M-109 lung carcinoma was kindly supplied by Bristol-Myers Squibb Co., Princeton, NJ. All the mice were purchased from Shizuoka Laboratory Animal Center, Hamamatu. The animals were given a pelleted food and water *ad libitum*. Two percent (w/v) homogenates of tumors were prepared from a 21-day-old M-109 lung carcinoma in RPMI-1640 medium (pH 7.4) with a glass homogenizer, and 0.1 ml of the homogenate was implanted subcutaneously into each of 6 CDF_1 mice on day 0. Percents of viable cells in the individual preparations (6 experiments) were in a range of 10.4 to 15.5% (trypan blue exclusion).

Drugs and administration Paclitaxel-based combination chemotherapy was examined with each of CDDP, JM-8, VP-16, MTX, ADM, CPM, MCNU and VLB in the present study. All drugs were administered intravenously to tumor-bearing mice in a volume of 0.01 ml/g body weight. We selected an administration schedule of consecutive daily injections for 5 days for paclitaxel, since this was one of the most effective treatment schedules according to our previous study.[11] CDDP, JM-8, VP-16, VLB and MTX were also given daily for 5 consecutive days, and CPM, MCNU and ADM were each given as a single dose. Combination schedules were as follows: (a) paclitaxel was given for the first 5 days (days 1–5) and one of the other drugs was given for the succeeding 5 days (days 6–10) or on day 6 only, (b) one of the other drugs were given for the first 5 days (days 1–5) or on day 1 only and paclitaxel was given for the succeeding 5 days (days 6–10) or (c) paclitaxel and one of 4 drugs (CDDP, JM-8, VP-16 and VLB) were given simultaneously for the first 5 days (days 1–5) (in this setting, paclitaxel was administered 2 h before the other drug). Paclitaxel was given to tumor-bearing mice at a dose of 24 mg/kg/day since this was the most effective dose according to our previous study.[11] Doses of the other drugs employed (Table I) were considered to be the most effective doses on the present treatment schedules and a half of those doses, which were estimated from our separate experiments with other murine tumors on the same treatment schedule (1 shot) or a different treatment schedule (days 1, 5 and 9). All drugs, except JM-8 and paclitaxel, were dissolved in saline. JM-8 was dissolved in distilled water. Paclitaxel was dissolved at 9.6 mg/ml in ethanol/Cremophor EL (1:1) solution, divided into 10 aliquots, and stored at 4°C for 10 days. These stock solutions were diluted to 2.4 mg/ml with 3 volumes of saline just before injection. The other drugs, except VLB, were also dissolved on the day of injection. VLB was dissolved at 0.12 and 0.06 mg/ml, divided each into 10 aliquots, and stored at 4°C for 10 days. Paclitaxel and the vehicle were administered over a period of about 1 min, and the other drugs and saline, within about 15 s.

Evaluation of antitumor activity For the survival experiments, the antitumor activity of drugs against tumor-bearing mice was assessed in terms of two parameters: (a) the mean survival time of the drug-treated mice, excluding long-term (80 days) survivors, versus saline-treated controls, expressed as percentage increase in life span (%ILS =T/C(treated/control)%−100) and (b) the incidence of long-term survivors. For the tumor-growth inhibition experiments, antitumor activity of drugs was assessed from the tumor-growth delay expressed as the treated minus control (T–C, days)[12] value; that is, the time required for the treatment-group tumors (the mean value) to reach 1,000 mg minus the time required for the control-group tumors to grow the same size. Tumor-free survivors were excluded from these calculations. The enhancement factor[8] was calculated in the combination groups, this factor being the T–C value of the group given a combination of drugs minus the T–C value of the group treated singly with the first drug of the combination, divided by the T–C value of the group treated singly with the second drug of the combination. Tumor sizes were measured on day 6, day 11, and two times a week thereafter, and tumor weights (mg) were calculated according to the formula: length (mm) × width $(mm)^2 \times 0.5$.[13] The criteria of effective antitumor activity were the same as those employed by Rose,[10] i.e., (a) more than 25% ILS of the mean survival time and (b) more than 4 days in T−C value.

Evaluation of toxicity The toxicity of drugs, given alone or in combination, to tumor-bearing mice was assessed in terms of two parameters: (a) toxic death and (b) maximum decrease in body weight. Death within 8 days after the last administration of drugs was considered to be toxic death.[8] Body weight of each tumor-bearing mouse was measured on day 0, day 6, day 11, and two times a week thereafter. The mean body weight of mice in each group was calculated by subtracting individual tumor weights. The maximum decrease (%) in body weight (BW) was calculated as follows: 100 × (the starting BW − the BW showing maximum decrease)/the starting BW. A maximum decrease in BW of 20% or more during the observation period before the beginning of weight loss due to cachexia (approximately 18 days after tumor implantation) was defined as toxic.[13]

Statistical analysis The results were analyzed for significance by Student's *t* test for the mean survival time of tumor-bearing mice and by Fisher's exact test for both the 80-day survival incidence and the toxic death incidence, and a *P* value of 0.05 or less was regarded as significant.

RESULTS

Single agent chemotherapy with paclitaxel and the vehicle against M-109 murine lung carcinoma We examined the antitumor activities of paclitaxel and 8 antitumor agents, given intravenously alone or in combination, against M-109 murine lung carcinoma implanted subcutaneously. In combination, drugs were administered on sequential and simultaneous treatment schedules. All the results are shown in Table I. Paclitaxel at 24 mg/kg/day on the schedule of days 1 to 5 (d1–5) reproducibly induced (Exp. 1–6) a significant increase in life span (ILS) of tumor-bearing mice as compared with saline-treated control mice. These antitumor activities were assessed as effective (>25% ILS) in the prolongation of the survival time of tumor-bearing mice and were reflected in a significant (>4 days T−C) tumor-growth delay of M-109 murine lung carcinoma. On the other hand, adverse effects were observed in the group treated with the vehicle (12.5% ethanol/12.5% Cremophor EL in saline) in 2 out of 6 experiments, i.e., there was a significant shortening in the mean survival time of tumor-bearing mice (Exp. 1) and one toxic death (Exp. 2). However, since none of the vehicle-treated groups showed any significant decrease in the body weight of tumor-bearing mice, the vehicle appeared to have little or no biological activity against tumor-bearing mice unless a rapid intravenous injection was performed. Paclitaxel itself (at 24 mg/kg/day) did not cause any significant decrease in body weight of tumor-bearing mice or toxic death (Exp. 1–6).

Antitumor activity and toxicity of paclitaxel-based combination chemotherapy on a sequential treatment schedule Antitumor activity and toxicity of the combination of paclitaxel and each of 8 antitumor agents on a sequential treatment schedule are shown in Table I (Exp. 1–3) and the therapeutic effects of typical combination groups are illustrated in Fig. 1. CDDP at either dose level did not show any significant antitumor activity when given alone (Exp. 1). However, the combination of paclitaxel followed by CDDP (2 mg/kg/day) showed a significant ($P<0.05$) ILS of tumor-bearing mice as compared with the group given paclitaxel alone. Moreover, this combination group showed a significant tumor-growth delay with an enhancing factor of 1.27; i.e., CDDP, given as the second drug, induced a tumor-growth delay 1.27-fold greater than the expected additive value of the two drugs. The reverse sequence of this combination caused toxic deaths of all mice. Thus, the antitumor activity of the combination of paclitaxel and CDDP was classified as a sequence-dependent type (Fig. 1A). Similarly, the combination of paclitaxel followed by JM-8 (10 mg/kg/day) induced a more-than-additive prolongation of the survival time of mice, while the combination of JM-8 (10 and 20 mg/kg/day) followed by paclitaxel caused toxic deaths of all mice (Exp. 1). Similar sequence-dependent antitumor activity and toxic death of mice were also observed in the combination of paclitaxel and MTX at 6 mg/kg/day (Exp. 2) or VP-16 at 18 mg/kg/day (Exp. 2).

On the other hand, VLB (0.6 mg/kg/day) showed an effective antitumor activity, i.e., 47% ILS and tumor-growth delay with a T−C value of 15.9 days, and showed no body weight loss (Exp. 3). The combination of paclitaxel followed by VLB (0.6 mg/kg/day) yielded 4 long-term survivors (without tumor) out of 6 tumor-bearing mice; this survival incidence being statistically significant ($P<0.05$) as compared with that (0/6) of the group given paclitaxel alone. A tumor growth was observed in one mouse of this group from 28 days after implantation of tumors. However, it should be stressed that, in this combination group, a transient paralysis of the lower limbs of all the tumor-bearing mice was observed around 9 days after initiation of treatment, and one mouse died from toxicity. The reverse sequence of this combination also showed a significant ($P<0.01$) ILS of tumor-bearing mice as compared with the group given paclitaxel alone. In this group, no sign of side effects and no toxic deaths were seen, and the maximum decrease in body weight was as low as 7%. Furthermore, this combination treatment induced a significant tumor-growth delay with a T−C value of 27.1 days, although the enhancing factor was 0.73. Thus, the antitumor activity of the combination of paclitaxel and VLB was classified as a sequence-independent type (Fig. 1B). Similarly, no marked sequence-dependent increase in toxic death of mice was observed with the combination of paclitaxel and MCNU at 20 mg/kg (Exp. 1), CPM at 150 mg/kg (Exp. 2) or ADM at 10 mg/kg (Exp. 3), and these combinations were also classified as being of sequence-independent type. Moreover, a more-than-additive therapeutic effect was observed with the combination of paclitaxel and MCNU.

Antitumor activity and toxicity of paclitaxel-based combination chemotherapy on a simultaneous treatment schedule Antitumor activity and toxicity of the combination of paclitaxel and CDDP, JM-8 or VP-16 (sequence-dependent type) and the combination of paclitaxel and VLB (sequence-independent type) were examined on a simultaneous treatment schedule. In each combination group, paclitaxel was administered 2 h before the other drug. As shown in Table I, CDDP, given alone, showed a dose-dependent antitumor activity (ILS and T−C) and toxicity (weight loss) (Exp. 4). The combination of paclitaxel and CDDP at 2 or 1 mg/kg/day

Table I. Toxicity and Antitumor Activity of Paclitaxel-based Combination Chemotherapy against M-109 Murine Lung Carcinoma

Exp. No.	Drug[a]	Schedule	Dose (mg/kg/day)	Toxic death[b]	Max. dec. (%) in BW[c]	MST[d]±SD[e] (days)	ILS[f] (%)	80-day survivors	T−C[g] (days)
1	Saline	d1–5	—	0/6	—	31.8±4.5	—	0/6	—
	Vehicle	d1–5	—	0/6	2	22.0±2.4[r]	−31	0/6	0
	PACL	d1–5	24	0/6	10	43.5±7.0[s]	37	0/6	12.3
	CDDP	d1–5	4	0/6	32	33.0±3.0	4	0/6	7.6
	CDDP	d1–5	2	0/6	11	28.0±6.4	−12	0/6	3.0
	PACL+CDDP	d1–5+d6–10	24+4	5/6[j]	41	22.7±19.8	−29	0/6	ND[h]
	CDDP+PACL	d1–5+d6–10	4+24	6/6[j]	27	9.8±0.8[r]	−69	0/6	ND
	PACL+CDDP	d1–5+d6–10	24+2	0/6	27	51.5±4.0[r, t]	62	0/6	16.1(1.27)[i]
	CDDP+PACL	d1–5+d6–10	2+24	6/6[k]	37	14.3±0.8[r]	−55	0/6	ND
	JM-8	d1–5	20	0/6	13	32.8±2.5	3	0/6	3.0
	JM-8	d1–5	10	0/6	6	24.8±6.6	−22	0/6	2.5
	PACL+JM-8	d1–5+d6–10	24+20	0/6	27	36.7±19.0	15	0/6	15.5(1.07)
	JM-8+PACL	d1–5+d6–10	20+24	6/6[l]	30	12.5±1.6[r]	−61	0/6	ND
	PACL+JM-8	d1–5+d6–10	24+10	0/6	20	48.5±3.9[r]	53	0/6	13.9(0.64)
	JM-8+PACL	d1–5+d6–10	10+24	4/6[m]	46	23.3±15.6	−27	0/6	ND
	MCNU	d1	40	2/6	32	8.0±2.3[r]	−75	2/6	ND
	MCNU	d1	20	0/6	5	36.8±12.9	16	0/6	10.4
	PACL+MCNU	d1–5+d6	24+40	3/6	37	24.0±26.4	−25	1/6	ND
	MCNU+PACL	d1+d6–10	40+24	6/6[n]	26	8.5±1.4[r]	−73	0/6	ND
	PACL+MCNU	d1–5+d6	24+20	0/6	18	60.6±11.0[r, t]	91	1/6	28.1(1.52)
	MCNU+PACL	d1+d6–10	20+24	0/6	28	54.5±5.5[r, t]	71	0/6	20.6(0.83)
2	Saline	d1–5	—	0/6	—	23.8±7.7	—	0/6	—
	Vehicle	d1–5	—	1/5	0	20.6±10.3	−13	0/5	−0.4
	PACL	d1–5	24	0/6	5	39.0±9.5[u]	64	0/6	11.5
	VP-16	d1–5	36	5/6	23	13.0±12.3	−45	0/6	ND
	VP-16	d1–5	18	0/6	16	33.3±5.6[u]	40	0/6	5.9
	PACL+VP-16	d1–5+d6–10	24+36	6/6	31	13.2±1.0[s]	−45	0/6	ND
	VP-16+PACL	d1–5+d6–10	36+24	6/6	23	8.3±0.5[r]	−65	0/6	ND
	PACL+VP-16	d1–5+d6–10	24+18	2/6	30	35.3±16.3	48	0/6	15.4(0.66)
	VP-16+PACL	d1–5+d6–10	18+24	6/6[o]	25	9.8±0.4[s]	−59	0/6	ND
	MTX	d1–5	12	0/6	5	30.2±1.7	27	0/6	0.8
	MTX	d1–5	6	0/6	4	23.3±6.8	−2	0/6	−0.1
	PACL+MTX	d1–5+d6–10	24+12	2/6	22	32.8±16.0	38	0/6	13.0(1.88)
	MTX+PACL	d1–5+d6–10	12+24	5/6[p]	29	16.8±13.9	−29	0/6	ND
	PACL+MTX	d1–5+d6–10	24+6	0/6	17	44.2±5.4[r]	86	0/6	13.2(1.70)
	MTX+PACL	d1–5+d6–10	6+24	3/6	24	30.2±18.5	27	0/6	13.4(1.17)
	CPM	d1	300	4/6	27	19.7±18.9	−17	0/6	ND
	CPM	d1	150	0/6	2	32.5±6.1	37	0/6	5.3
	PACL+CPM	d1–5+d6	24+300	6/6	26	10.0±0.6[s]	−58	0/6	ND
	CPM+PACL	d1+d6–10	300+24	6/6	20	8.3±0.5[r]	−65	0/6	ND
	PACL+CPM	d1–5+d6	24+150	1/6	20	37.0±13.5	55	0/6	13.3(0.34)
	CPM+PACL	d1+d6–10	150+24	1/6	24	38.0±16.2	60	0/6	16.1(0.94)
3	Saline	d1–5	—	0/6	—	27.5±6.3	—	0/6	—
	Vehicle	d1–5	—	0/6	0	29.8±6.9	8	0/6	0.6
	PACL	d1–5	24	0/6	10	48.8±8.9[r]	77	0/6	15.4
	ADM	d1	10	0/6	2	41.0±6.9[s]	49	0/6	11.1
	ADM	d1	5	0/6	0	37.0±6.3[u]	35	0/6	5.2
	PACL+ADM	d1–5+d6	24+10	0/6	22	50.3±9.3[r]	83	0/6	21.0(0.50)
	ADM+PACL	d1+d6–10	10+24	0/6	25	57.0±8.8[r]	107	0/6	27.9(1.09)
	PACL+ADM	d1–5+d6	24+5	0/6	20	46.0±4.6[r]	67	0/6	18.6(0.62)
	ADM+PACL	d1+d6–10	5+24	0/6	18	46.7±7.0[r]	70	0/6	19.1(0.90)
	VLB	d1–5	1.2	4/6	14	22.2±22.1	−19	0/6	ND
	VLB	d1–5	0.6	0/6	0	40.3±7.5[s]	47	0/6	15.9
	PACL+VLB	d1–5+d6–10	24+1.2	6/6	30	10.8±1.3[r]	−61	0/6	ND
	VLB+PACL	d1–5+d6–10	1.2+24	5/6	14	17.2±21.0	−37	0/6	ND
	PACL+VLB	d1–5+d6–10	24+0.6	1/6	17	35.0±36.8	27	4/6[w]	28.7[x](0.84)
	VLB+PACL	d1–5+d6–10	0.6+24	0/6	7	62.3±5.2[r, v]	127	0/6	27.1(0.73)
4	Saline	d1–5	—	0/6	—	24.7±4.2	—	0/6	—
	Vehicle	d1–5	—	0/6	0	26.2±7.1	6	0/6	0.2
	PACL	d1–5	24	0/6	9	45.0±4.9[r]	82	0/6	10.5
	CDDP	d1–5	4	0/6	26	30.7±3.8[t]	24	0/6	7.9
	CDDP	d1–5	2	0/6	10	28.8±7.1	17	0/6	2.2
	CDDP	d1–5	1	0/6	7	28.3±6.6	15	0/6	1.6
	PACL+CDDP	d1–5+d1–5	24+4	5/6[j]	18	15.5±19.4	−37	0/6	ND

Table I. (Continued)

Exp. No.	Drug[a)]	Schedule	Dose (mg/kg/day)	Toxic death[b)]	Max. dec. (%) in BW[c)]	MST[d)]±SD[e)] (days)	ILS[f)] (%)	80-day survivors	T−C[g)] (days)
	PACL+CDDP	d1–5+d1–5	24+2	0/6	21	47.2±2.9[r)]	91	0/6	13.8(1.50)
	PACL+CDDP	d1–5+d1–5	24+1	0/6	17	46.5±1.8[r)]	88	0/6	13.8(2.06)
	VLB	d1–5	1.2	0/6	0	39.5±9.4[s)]	60	0/6	11.5
	VLB	d1–5	0.6	0/6	0	40.3±9.6[s)]	63	0/6	11.8
	VLB	d1–5	0.3	0/6	0	37.7±9.2[t)]	53	0/6	6.4
	PACL+VLB	d1–5+d1–5	24+1.2	2/6	19	34.0±20.7	38	0/6	13.9(0.30)
	PACL+VLB	d1–5+d1–5	24+0.6	2/6	14	32.5±19.8	32	0/6	13.2(0.23)
	PACL+VLB	d1–5+d1–5	24+0.3	1/6	8	38.8±15.6	57	0/6	12.7(0.34)
5	Saline	d1–5	—	0/6	—	30.0±4.1	—	0/6	—
	Vehicle	d1–5	—	0/6	0	29.7±6.0	−1	0/6	1.3
	PACL	d1–5	24	0/6	10	46.3±9.4[s)]	54	0/6	14.5
	JM-8	d1–5	20	0/6	7	29.3±0.9	−2	0/6	2.0
	JM-8	d1–5	10	0/6	1	29.0±4.2	−3	0/6	1.9
	JM-8	d1–5	5	0/6	0	29.7±5.6	−1	0/6	0.9
	PACL+JM-8	d1–5+d1–5	24+20	2/6	25	30.0±19.8	0	0/6	13.9
	PACL+JM-8	d1–5+d1–5	24+10	1/6	16	38.0±17.8	27	0/6	11.9
	PACL+JM-8	d1–5+d1–5	24+5	0/6	11	46.8±4.7[r)]	56	0/6	14.6(0.11)
6	Saline	d1–5	—	0/6	—	26.0±6.7	—	0/6	—
	Vehicle	d1–5	—	0/6	0	28.7±5.4	10	0/6	−2.0
	PACL	d1–5	24	0/6	8	39.2±4.3[s)]	51	0/6	9.7
	VP-16	d1–5	36	6/6	15	8.0±1.1[s)]	−69	0/6	ND
	VP-16	d1–5	18	0/6	14	28.8±7.4	11	0/6	4.1
	VP-16	d1–5	9	0/6	4	34.5±5.5	33	0/6	2.0
	PACL+VP-16	d1–5+d1–5	24+36	6/6	19	5.8±1.2[s)]	−78	0/6	ND
	PACL+VP-16	d1–5+d1–5	24+18	6/6[o)]	20	6.8±1.0[s)]	−74	0/6	ND
	PACL+VP-16	d1–5+d1–5	24+9	4/6[q)]	16	20.2±21.6	−22	0/6	ND

a) Male CDF_1 mice were inoculated subcutaneously with a 2% homogenate of M-109 murine lung carcinoma (0.1 ml/mouse) on day 0, and animals were divided randomly into test groups consisting of 6 mice per group. Drugs and a vehicle were administered intravenously on the indicated schedules of sequential combinations (Exp. 1–3). For simultaneous combinations (Exp. 4–6), paclitaxel was administered 2 h before each of the other drugs. Vehicle: 12.5% ethanol/12.5% Cremophor EL in saline, PACL: paclitaxel, CDDP: cisplatin, JM-8: carboplatin, MCNU: ranimustine, VP-16: etoposide, MTX: methotrexate, CPM: cyclophosphamide, ADM: adriamycin, and VLB: vinblastine.
b) The number of mice that died from toxicity within 8 days after the last administration of drugs/the total number of mice.
c) Maximum decrease (%) in body weight (BW): 100×(the starting BW−the BW showing maximum decrease)/the starting BW. The maximum values during the observation period before beginning of weight loss due to cachexia are shown.
d) MST: mean survival time of tumor-bearing mice excluding 80-day survivors (all these mice were tumor-free on day 140).
e) SD: standard deviation.
f) ILS: increase in life span of drug-treated groups over saline-treated control group.
g) T−C (days): time required for the treatment-group tumors (the mean values) to reach 1,000 mg minus the time required for the control-group tumors to grow the same size.
h) ND: not determined due to toxic death of more than half of tumor-bearing mice in the group.
i) Enhancement factor: the T−C value of the group given a combination of drugs minus the T−C value of the group treated singly with the first drug of the combination, divided by the T−C value of the group treated singly with the second drug of the combination.
j) Significantly different from CDDP at 4 mg/kg/day group, $P<0.01$ by Fisher's exact test.
k) Significantly different from CDDP at 2 mg/kg/day group, $P<0.01$ by Fisher's exact test.
l) Significantly different from JM-8 at 20 mg/kg/day group, $P<0.01$ by Fisher's exact test.
m) Significantly different from JM-8 at 10 mg/kg/day group, $P<0.05$ by Fisher's exact test.
n) Significantly different from MCNU at 40 mg/kg group, $P<0.05$ by Fisher's exact test.
o) Significantly different from VP-16 at 18 mg/kg/day group, $P<0.01$ by Fisher's exact test.
p) Significantly different from MTX at 12 mg/kg/day group, $P<0.01$ by Fisher's exact test.
q) Significantly different from VP-16 at 9 mg/kg/day group, $P<0.05$ by Fisher's exact test.
r) Significantly different from control group, $P<0.001$ by Student's *t* test.
s) Significantly different from control group, $P<0.01$ by Student's *t* test.
t) Significantly different from paclitaxel group, $P<0.05$ by Student's *t* test.
u) Significantly different from control group, $P<0.05$ by Student's *t* test.
v) Significantly different from paclitaxel group, $P<0.01$ by Student's *t* test.
w) Significantly different from paclitaxel group, $P<0.05$ by Fisher's exact test.
x) A result for one mouse is shown since no tumor growth was seen in 4 out of 5 tumor-bearing mice.

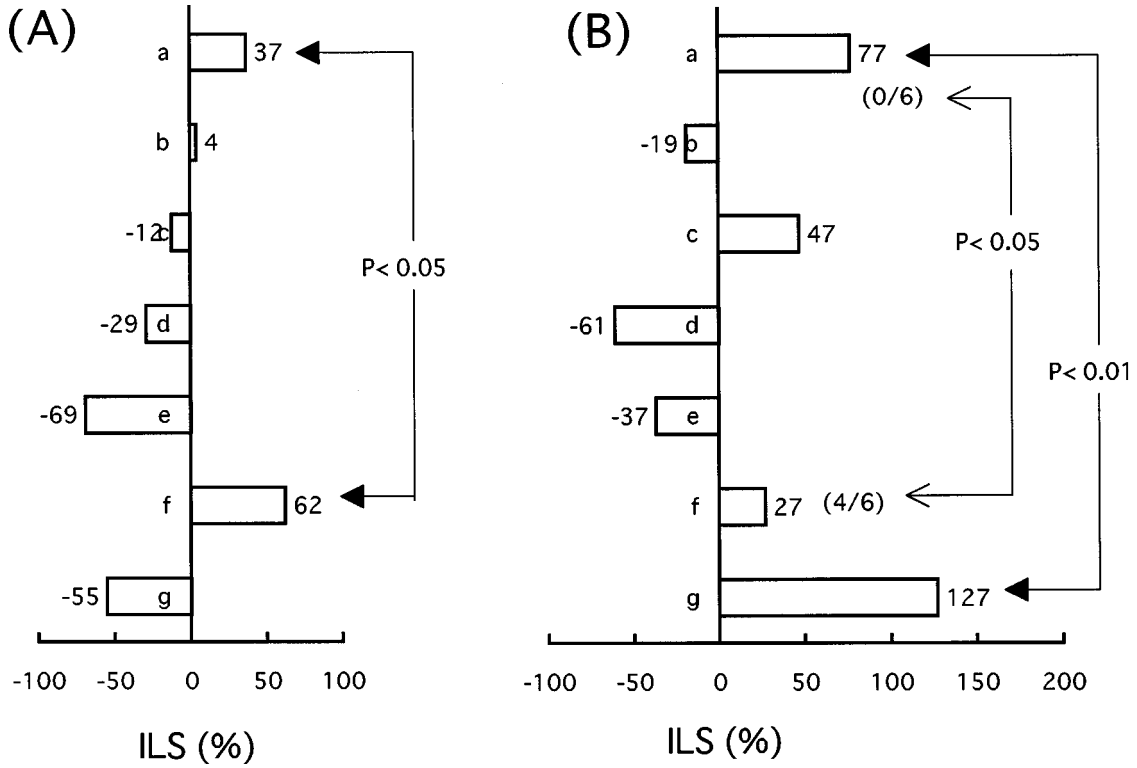

Fig. 1. Sequence-dependent (A) and -independent (B) antitumor activity of paclitaxel-based combination chemotherapy against M-109 murine lung carcinoma. (A) The combination of paclitaxel and cisplatin (CDDP). a, paclitaxel (24 mg/kg/day) alone; b, CDDP (4 mg/kg/day) alone; c, CDDP (2 mg/kg/day) alone; d, paclitaxel (24 mg/kg/day) followed by CDDP (4 mg/kg/day); e, CDDP (4 mg/kg/day) followed by paclitaxel (24 mg/kg/day); f, paclitaxel (24 mg/kg/day) followed by CDDP (2 mg/kg/day); g, CDDP (2 mg/kg/day) followed by paclitaxel (24 mg/kg/day). (B) The combination of paclitaxel and vinblastine (VLB). a, paclitaxel (24 mg/kg/day) alone; b, VLB (1.2 mg/kg/day) alone; c, VLB (0.6 mg/kg/day) alone; d, paclitaxel (24 mg/kg/day) followed by VLB (1.2 mg/kg/day); e, VLB (1.2 mg/kg/day) followed by paclitaxel (24 mg/kg/day); f, paclitaxel (24 mg/kg/day) followed by VLB (0.6 mg/kg/day); g, VLB (0.6 mg/kg/day) followed by paclitaxel (24 mg/kg/day). → indicates a statistically significant difference in the survival time of tumor-bearing mice, and ⇒ indicates that in the 80-day survival incidence.

showed a slightly higher ILS (91% or 88%) of tumor-bearing mice as compared with the group given paclitaxel alone (82%). Moreover, these combination groups showed a significant tumor-growth delay with an enhancing factor of 1.50 and 2.06, respectively. The maximum weight loss was only marginal (21% or 17%) and no toxic death was seen. The combination of paclitaxel and JM-8 at 5 mg/kg/day showed a similar level of ILS (56%) or T−C value (14.6 days) to that of the paclitaxel-treated group (54% or 14.5 days), and no increase in toxicity (weight loss or toxic death) was observed (Exp. 5). The maximum tolerable dose (MTD) of VP-16 on the schedule of days 1–5 appeared to be 18 mg/kg/day (Exp. 2 and 6). The combination of paclitaxel and VP-16 at this dose level caused toxic deaths of all the mice, and the combination of paclitaxel and VP-16 at 9 mg/kg/day caused 4 toxic deaths out of 6 mice (Exp. 6). Also, the MTD of VLB on the schedule of days 1–5 seemed to be 0.6 mg/kg/day (Exp. 3). The combination of paclitaxel and VLB at this dose level caused 2 toxic deaths out of 6 mice, and the combination of paclitaxel and VLB at 0.3 mg/kg/day caused one toxic death out of 6 mice (Exp. 4). Moreover, the antitumor activities (ILS and T−C) of the combination of paclitaxel and VLB at these two dose levels were far lower than those on the sequential treatment schedule (Exp. 3).

DISCUSSION

We carried out the present study in an *in vivo* setting so as to ascertain the antitumor activity and toxicity of the combination of paclitaxel and each of 8 antitumor agents currently in clinical use, i.e., CDDP, JM-8, VP-16, MTX, ADM, CPM, MCNU and VLB, against M-109 murine lung carcinoma implanted subcutaneously. We also per-

formed these experiments to determine whether schedule-dependent antitumor activity and toxicity would be observed in paclitaxel-based combination chemotherapy. Our results demonstrated that paclitaxel-based combination chemotherapy could be divided into two types with respect to the sequence-dependency: a sequence-dependent type (Fig. 1A) and a sequence-independent type (Fig. 1B). The combination of paclitaxel and CDDP, JM-8, VP-16 or MTX belonged to the former type; i.e., treatment with paclitaxel followed by one of these drugs elicited a favorable antitumor activity against M-109 murine lung carcinoma, but treatment with the same drugs in the reverse sequence resulted in toxic outcomes (Table I, Exp. 1 and 2). On the other hand, the combination of paclitaxel and VLB, MCNU, ADM or CPM belonged to the latter type; i.e., treatment with the 2 drugs in either sequence induced a similar level of favorable therapeutic effects without a serious increase in toxic death of mice (Exp. 1–3).

Interestingly, the present study also showed that MTD of each drug in the sequence-dependent combinations could not be combined with paclitaxel at 24 mg/kg/day (the maximum effective dose) because of increased toxic deaths (CDDP at 4 mg/kg/day, VP-16 at 18 mg/kg/day and MTX at 12 mg/kg/day) or a decreased survival time (JM-8 at 20 mg/kg/day) (Exp. 1 and 2), while the MTD of each drug of the sequence-independent type could be combined with paclitaxel without an excessive increase in toxicity (Exp. 1–3). On the simultaneous treatment schedule, the combination of paclitaxel and CDDP, JM-8, VLB or VP-16 at MTD caused toxic deaths, which were especially prominent in the combination with VP-16 (Exp. 4–6).

Antitumor activity of combinations of paclitaxel and other clinically useful agents *in vivo* has been reported in only a limited number of studies.[8–11] Rose[10] examined the combination effect of paclitaxel and CDDP, VP-16, CPM or ADM against M-109 murine lung carcinoma implanted subcutaneously. Paclitaxel was administered on days 1–5 and CDDP, VP-16, CPM or ADM on both day 1 and day 5 to tumor-bearing mice. Thus, he failed to observe the sequence-dependent antitumor activity and/or toxicity in the combination of paclitaxel and CDDP. Milross *et al*.[8] demonstrated sequence-dependent antitumor activity (tumor growth delay) and toxicity (mortality) of the combination of paclitaxel and CDDP *in vivo* against a murine ovarian carcinoma (OCa-I) and they concluded that, when these 2 agents are given in combination, the sequence of choice is paclitaxel followed by CDDP. These observations are consistent with those of the present study.

A favorable combination effect (more-than-additive therapeutic effect) was observed with the combination of paclitaxel and CDDP (Exp. 1), JM-8 (Exp. 1), MCNU (Exp. 1) or VLB (Exp. 3). The mechanisms of individual favorable combination effects (and also those of unfavorable toxic combination effects) are of interest. Among them, the mechanism through which beneficial therapeutic effects were elicited by the combination of paclitaxel and VLB is of special interest. Although both agents target microtubules and interfere with mitotic spindle function, their mechanisms of action are distinct. VLB acts to depolymerize microtubules[6] and paclitaxel acts to stabilize polymerized tubulin into nonfunctional microtubule bundles.[5] Speicher *et al*.[14] found no additive cytotoxicity *in vitro* against the human prostate carcinoma cell line DU 145 with the combination of paclitaxel and VLB. Furthermore, Chou *et al*.[15] found an antagonistic interaction *in vitro* against the human teratocarcinoma cell line 833K with the combination of paclitaxel and vincristine. These observations presumably reflect the respective mechanisms of action. On the other hand, Knick *et al*.[9] showed *in vivo* that the LD_{10} (dose lethal to 10% of the mice) of vinorelbine tartrate, a hemisynthetic vinca alkaloid, increased approximately 2.5-fold in the presence of paclitaxel (given 1 h after vinorelbine tartrate) and allowed otherwise lethal vinorelbine tartrate doses to be administered safely, which may have contributed to the enhanced antitumor efficacy of the combinations against P388 murine leukemia. In the present study, we also observed a favorable *in vivo* therapeutic effect of the combination of paclitaxel and VLB (Exp. 3). However, we did not observe the toxicity-reducing activity of paclitaxel in the combination with VLB at any treatment schedule, but noted favorable therapeutic effects with sequential treatment schedules. This may be due to the difference of combination schedule employed, since the time interval between doses of vinorelbine tartrate and paclitaxel was shown to be critical to the therapeutic outcome of this combination.[9] Possibly, the cellular interactions observed between paclitaxel and either VLB or vincristine that cause antagonism *in vitro* are not expressed *in vivo* to the same degree or in the same manner. Jordan *et al*.[16] showed that, at submicromolar concentrations, paclitaxel appears to block mitosis and inhibit cell proliferation by inhibiting the dynamics of spindle microtubules in a manner similar to VLB. It is conceivable that the intracellular levels of paclitaxel and VLB may determine the mechanism by which these two drugs interact. In such a case, the finding[17] that paclitaxel did not compete with VLB for binding to tubulin is of interest, and might be relevant to the mechanisms of favorable interaction of these two agents.

Treatment with paclitaxel followed by CDDP (JM-8 also) elicited a favorable antitumor activity against M-109 murine lung carcinoma, but treatment with the same drugs in the reverse sequence resulted in toxic outcomes (Exp. 1). Christen *et al*.[18] showed that pretreatment of 2008 human ovarian carcinoma cells *in vitro* with paclitaxel increased CDDP accumulation in a dose-dependent manner. In addition, Liebmann *et al*.[19] showed that exposure

of human lung A549 and breast MCF-7 adenocarcinoma cells to 100 n*M* paclitaxel for 24 h blocked a majority of the cells into the G_2/M phase. Kubota *et al.*[20] revealed that the maximum concentrations of paclitaxel in plasma and tumor of mice after a single administration of paclitaxel (20 mg/kg) were 1,800 n*M* and 2,000 n*M*, respectively. Thus, viable cells emerging from paclitaxel-induced G_2/M block encounter the subsequent CDDP in the G_1 phase, during which they are most sensitive to the effects of CDDP.[21] These factors may contribute to the favorable therapeutic effect of treatment with paclitaxel followed by CDDP. On the other hand, Milross *et al.*[8] demonstrated that a single treatment with CDDP (10 mg/kg) followed by paclitaxel (40 mg/kg) caused 11 toxic deaths among 47 mice, compared with only 2 deaths among 47 mice in the reverse sequence. Similarly, in the present study, 6 of 6 mice died on treatment with CDDP (2 mg/kg/day) given for days 1–5 before paclitaxel (24 mg/kg/day) given for days 6–10, compared with 0 of 6 mice on the reverse sequence (Exp. 1). It is possible that the toxicity of this sequence arises from a CDDP-induced reduction of paclitaxel clearance, as suggested in an earlier clinical study conducted by Rowinsky *et al.*[22]

CPM and MCNU are alkylating agents and their cell cycle activity is considered to be cell cycle-phase nonspecific.[23] Thus, it is reasonable that the treatment sequence of paclitaxel and these alkylators may not be critical for the therapeutic outcome. On the other hand, MTX has an S-phase specific but self-limiting action.[23] Therefore, the cell cycle blockade in the G_2/M phase by preceding paclitaxel may interfere with the subsequent cytotoxicity of MTX. In the reverse sequence, preceding MTX, which inhibits DNA, RNA and protein synthesis, may destroy the proliferative integrity only of cells in S-phase, and, because of effects on RNA and protein synthesis, may slow down the movement of G_2, M and G_1 cells into S-phase (i.e., the rate of cell killing is self-limiting).[23] Thus, paclitaxel after MTX may exhibit enhanced cytotoxicity.

Finally, ADM and VP-16 are topoisomerase II inhibitors.[24, 25] Hahn *et al.*[26] observed less-than-additive cytotoxicity *in vitro* against the human breast cancer cell line MCF7 and the human lung adenocarcinoma cell line A549 with the combination of paclitaxel and ADM, as well as the combination of paclitaxel and VP-16. Thus, it remains unclear why ADM belongs to the sequence-independent group, while VP-16 is sequence-dependent.

Many complex *in vivo* cellular interactions may be involved in the enhanced therapeutic activity and/or increased toxicity of the combinations of paclitaxel and each of the 8 antitumor agents observed in the present study. Thus, the mechanisms of the individual favorable and unfavorable combination effects should be further studied. However, it is important to emphasize that there is a possibility of paclitaxel combinations exhibiting unexpected toxicity which depends on the schedule employed.

On the basis of our previous study,[11] we chose an administration schedule of consecutive daily injections for 5 days (days 1–5) for paclitaxel since paclitaxel, on this treatment schedule, induced a more significant tumor-growth delay than it did on the schedule of q4d×3 (days 1, 5 and 9) or a single injection (day 1) against M-109 murine lung carcinoma. Furthermore, CDDP, JM-8, VP-16, VLB and MTX were also given daily for 5 consecutive days, and CPM, MCNU and ADM were each given as a single dose. Therefore, the drug administration schedules used here may not be directly applicable to the clinic, but our results on the nature of the sequence-dependency in paclitaxel-based combination chemotherapy should be useful in the design of clinical trials.

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