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Original Paper

Sequence-dependent Antitumour Efficacy of Combination Chemotherapy of Nedaplatin, a Novel Platinum Complex, with 5-Fluorouracil in an *in vivo* Murine Tumour Model

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The antitumour efficacy of a sequential combination of nedaplatin (NDP) and 5-fluorouracil (5-FU) was evaluated using Lewis lung carcinoma *in vivo*. NDP was developed as a second generation platinum complex. Because it has greater antitumour activity and lower nephrotoxicity than cisplatin (CDDP), we also compared the antitumour activity of NDP plus 5-FU with that of CDDP plus 5-FU. A fixed 5-FU dose was injected daily for 5 days and increasing doses of either NDP or CDDP were injected once via the tail vein into the Lewis lung carcinoma-implanted mice. The sequential administration of either NDP or CDDP prior to 5-FU (NF or CF therapy) resulted in severe body weight loss followed by the death of the tumour-bearing mice when the high-dose of NDP or CDDP was administered. In contrast, the sequential administration of 5-FU prior to NDP or CDDP (FN or FC therapy) resulted in synergistically enhanced inhibition of tumour growth and prolonged survival in comparison with NDP, CDDP or 5-FU monotherapy. At the high-dose of NDP in FN therapy, a reduction of tumour size and long-term tumour-free survival were frequently observed. The survival effect of the combinations of NDP with 5-FU was superior to those of the combination of CDDP with 5-FU. In conclusion, the sequence-dependent antitumour efficacy and toxicity of the combination of NDP or CDDP with 5-FU was demonstrated in this study, and FN therapy appeared to be the most efficient regimen as a clinical therapy. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: platinum complex, nedaplatin, cisplatin, 5-FU, combination chemotherapy
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INTRODUCTION

NEDAPLATIN (NDP) was selected from a series of platinum analogues based on its pronounced antitumour activity against solid tumours, including Colon 38 colon carcinoma, Lewis lung carcinoma, B16 melanoma, Walker 256 carcinoma and MX-1 breast cancer, with lower nephrotoxicity than cisplatin (CDDP) in preclinical studies [1–5]. In clinical phase II studies, NDP showed prominent efficacy against lung [6, 7], head and neck [8], testicular [9], and gynaecological [10] cancers. The chemical structure of NDP is shown in Figure 1.

Recently, both preclinical and clinical studies have demonstrated that the combination of CDDP and 5-fluorouracil (5-FU) results in synergistic antitumour activity on various cancers [11–15]. However, the optimal administration schedule for this combination still remains to be determined.

In the present study, we compared *in vivo* antitumour efficacy of the sequential administration of 5-FU prior to NDP (FN therapy) with its reverse sequence (NF therapy). We also compared the therapeutic efficacy of NDP and CDDP combined with 5-FU.

MATERIALS AND METHODS

Animals

The BDF1 and C57BL/6 mice (female, 7–9 weeks old) used in this study were produced in our breeding colony and maintained in a specific pathogen-free facility at Aburahi Laboratory (Shionogi and Co. Ltd, Kohga, Japan).

Tumour

Lewis murine lung carcinoma was obtained from the National Cancer Institute (NCI, Bethesda, Maryland,

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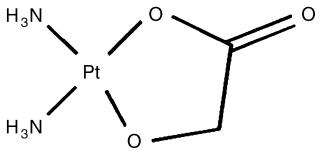

Figure 1. Chemical structure of nedaplatin.

U.S.A.) and was maintained by serial subcutaneous (s.c.) transplantation as tumour fragments in C57BL/6 mice.

Drugs

NDP (Lot:BF-4003) was synthesised at Shionogi and Co. Ltd (Osaka, Japan). CDDP and 5-FU were purchased from Nippon Kayaku (Tokyo, Japan) and Kyowa Hakko (Tokyo, Japan), respectively. All drugs were dissolved in saline immediately before use.

In vivo *therapeutic experiments*

The experimental procedure has been described previously [4, 17]. All experiments consisted of 6–10 mice per group. On day 0, a tumour fragment (8 mm^3) of Lewis lung carcinoma was implanted s.c. into the back of BDF1 mice. 5-FU was injected intravenously (i.v.) daily for 5 days from day 3. NDP or CDDP was administered by a single i.v. injection on day 3 for NF or CF therapy and on day 7 for FN or FC therapy. The doses of drugs used were 44, 22, and 11 mg/kg for NDP; 12, 6, and 3 mg/kg for CDDP; and 13.1 mg/kg for 5-FU. The maximum tolerated doses (MTDs) of NDP, CDDP and 5-FU were 44, 12, and 210 mg/kg, respectively, in the conventional mice. All studies were performed with an approval of Shionogi Animal Care and Use Committee.

Evaluation of antitumour efficacy

Tumour size, body weight and survival were assessed throughout each experiment up to day 45. The endpoint of survival was judged by the onset of moribundity, such as hypoactivity or hypothermia, and the mice were then sacrificed. The growth inhibitory effect and prolonged survival were estimated using the treated/control ratio (T/C) and increased life span (ILS%) against controls, as described previously [4]. For the evaluation of combination therapies, the interaction index (I.I.) and the combination index (C.I.) were used [18, 19]. The I.I. was calculated using the T/C value by the method of Berenbaum [19] with I.I. = Ac/Ae + Bc/Be, where Ac, Bc are the doses of drug A and drug B for the combination; Ae, Be are the doses of drug A and drug B required alone to give an equivalent T/C as that in the combination.

An I.I. less than 1 indicated synergy (i.e. the effect of the combination was greater than that expected from the additive effects of the component agent), an I.I. equal to 1 indicated additivity and an I.I. greater than 1 indicated antagonism.

The C.I. was calculated using ILS% values as follows [18]: C.I. = ILS% of drug A with drug B/(ILS% of drug A + ILS% of drug B).

A C.I. greater than 1 indicated synergy, a C.I. equal to 1 indicated additivity and a C.I. less than 1 indicated antagonism.

In this study, the statistical significance in comparison with the non-treated group or between treated groups was evaluated using Bonferroni's test or Dunnett's test, respectively [20, 21].

Haematotoxicity study

Blood (0.5 ml) was collected from the portal vein of anaesthetised BDF1 mice. Nucleated bone marrow cells (BMC) were collected from the right femur. The number of white blood cells, platelets, red blood cells and BMC were counted by an automatic cell counter (K-1000 and CDA-500, Sysmex, Kobe, Japan).

RESULTS

NF and CF therapy against Lewis lung carcinoma

As shown in Table 1, treatment with the high-dose of NDP or CDDP prior to 5-FU resulted in increased toxicity and all the treated mice died by day 11. The treatment with lower doses of NDP or CDDP following 5-FU showed an enhanced growth inhibitory activity compared with the use of NDP, CDDP or 5-FU alone. This activity did contribute to the survival of the tumour-bearing animals in NF therapy,

Table 1. *Antitumour efficacy of nedaplatin (NDP) or cisplatin (CDDP) followed by* 5*-fluorouracil (*5*-FU) against Lewis lung carcinoma*

Group	Total dose (mg/kg) NDP†	CDDP‡	5-FU§	Tumour volume¶ (mm^3 ± S.D.)	T/C¶	I.I.	Survival days (Mean ± S.D.)	ILS%	C.I.
Untreated control	0	0	0	3218 ± 1253			15.8 ± 3.0		
5-FU only	0	0	65.5	959 ± 636**	0.30		20.8 ± 2.0*	32	
NDP only	11	0	0	2718 ± 667	0.84		16.7 ± 0.9	6	
	22	0	0	2255 ± 942	0.70		17.5 ± 1.3	11	
	44	0	0	417 ± 444**	0.13		22.2 ± 3.9**	41	
NF therapy	11	0	65.5	503 ± 236**	0.16	1.01	21.2 ± 2.7*	34	0.89
	22	0	65.5	55 ± 69**	0.02	0.86‖	23.5 ± 6.9**	49	1.13‖
	44	0	65.5	Toxic death			9.3 ± 0.7**	− 41	− 0.56
CDDP only	0	3	0	2933 ± 816	0.91		18.2 ± 1.7	15	
	0	6	0	2583 ± 965	0.80		17.7 ± 1.8	12	
	0	12	0	1317 ± 333**	0.41		18.5 ± 1.4	17	
CF therapy	0	3	65.5	949 ± 493**	0.29	1.34	17.8 ± 5.6	13	0.28
	0	6	65.5	254 ± 240**	0.08	0.79‖	17.0 ± 6.3	8	0.18
	0	12	65.5	Toxic death			9.2 ± 0.4**	− 42	− 0.86

*$P < 0.05$; †i.v.×1 (day 3); ‡i.v.×1 (day 3); §i.v.×5 (days 3–7); ¶on day 12; **$P < 0.01$ for no treatment by Bonferroni's test; ‖, synergistic effect; S.D., standard deviation; T/C, treated/control ratio; I.I., interaction index; ILS, increased life span; C.I., combination index; i.v., intravenous.

but not in CF therapy (maximum ILS% in the NF and CF therapy were 49% ($P < 0.01$) and 13% (not significant), respectively).

FN and FC therapy against Lewis lung carcinoma

In contrast to NF and CF therapy, FN and FC therapy demonstrated more potent antitumour activity without lethal toxicity, as shown in Table 2. The interactions of the two drugs' efficacy on growth inhibition and survival were analysed by calculating the I.I. and the C.I., which indicated that the growth inhibitory activity and survival effect were synergistic in both FN and FC therapy. It is noteworthy, however, that the enhanced antitumour activity contributed to the survival outcomes only in the FN therapy. Three of six mice treated with the high-dose of NDP with 5-FU in the FN therapy survived more than 45 days (three times longer than the mean survival period of the non-treated mice) without any recurrent tumour. No long-term survivor was found in either the single treatment groups or the group treated with the FC therapy. ILS% in the FN therapy was significantly greater than that of FC therapy at high-dose ($P < 0.01$).

Simultaneous administration of NDP and 5-FU

We have previously demonstrated that intermittent high dose administration of NDP is the most efficient schedule for obtaining the maximum antitumour activity in an experimental study [22]. Thus, we used the schedule of three times simultaneous administration of NDP and 5-FU at 4 day intervals. As shown in Table 3, high-dose treatment with NDP only improved survival (79% versus 41% in Table 1 or 54% in Table 2). However, the survival periods in the combination therapy of high-dose platinum and 5-FU were rather shortened because of increased toxicity (− 35% in NDP + 5-FU and − 21% in CDDP + 5-FU).

Table 2. Antitumour efficacy of 5-fluorouracil (5-FU) followed by nedaplatin (NDP) or cisplatin (CDDP) against Lewis lung carcinoma

Group	Total dose (mg/kg) NDP†	CDDP‡	5-FU§	Tumour volume¶ ($mm^3 \pm$ S.D.)	T/C	I.I.	Survival days (Mean ± S.D.)	ILS%	C.I.
Untreated control	0	0	0	3277 ± 1236			16.2 ± 1.6		
5-FU only	0	0	65.5	1277 ± 304**	0.39		19.0 ± 2.5	17	
NDP only	11	0	0	2972 ± 628	0.91		17.0 ± 1.1	5	
	22	0	0	1683 ± 807**	0.51		20.2 ± 2.0*	25	
	44	0	0	1013 ± 340**	0.31		25.0 ± 1.1**	54	
FN therapy	11	0	65.5	248 ± 56**	0.08	0.80‖	24.7 ± 2.1**	52	2.36‖
	22	0	65.5	172 ± 64**	0.05	0.93‖	25.0 ± 1.7**	54	1.29‖
	44	0	65.5	11 ± 5**	0.003	1.16	39.0 ± 7.2**	> 141	2.31‖
CDDP only	0	3	0	2497 ± 564	0.76		18.2 ± 1.0	12	
	0	6	0	2192 ± 270**	0.67		20.3 ± 3.2*	25	
	0	12	0	1567 ± 646**	0.48		23.8 ± 3.5**	47	
FC therapy	0	3	65.5	495 ± 153**	0.15	0.87‖	22.0 ± 2.0**	36	1.24‖
	0	6	65.5	301 ± 29**	0.09	0.82‖	22.2 ± 5.5**	37	0.88
	0	12	65.5	17 ± 23**	0.005	0.76‖	29.7 ± 2.2**	83	1.30‖

*$P < 0.05$; †i.v.×1 (day 7); ‡i.v.×1 (day 7); §i.v.×5 (days 3–7); ¶on day 12; **$P < 0.01$ for no treatment by Bonferroni's test; ‖, Synergistic effect; S.D., standard deviation; T/C, treated/control ratio; I.I., interaction index; ILS, increased life span; C.I., combination index.

Table 3. Antitumour efficacy of nedaplatin (NDP) or cisplatin (CDDP) with 5-fluorouracil (5-FU) against Lewis lung carcinoma

Group	Total dose (mg/kg) NDP†	CDDP‡	5-FU§	Tumour volume¶ ($mm^3 \pm$ S.D.)	T/C	Survival days (Mean ± S.D.)	ILS%	C.I.
Untreated control	0	0	0	2170 ± 814		17.3 ± 2.76		
5-FU only	0	0	105	1089 ± 420**	0.50	21.8 ± 2.9	26	
NDP only	16.5	0	0	1535 ± 616	0.71	19.2 ± 2.9	11	
	33	0	0	961 ± 399**	0.44	21.5 ± 0.8	24	
	66	0	0	546 ± 316**	0.25	31.0 ± 1.7**	79	
NDP + 5-FU	16.5	0	105	673 ± 296**	0.31	22.7 ± 3.9	31	0.84
	33	0	105	514 ± 435**	0.24	21.2 ± 10.7	23	0.46
	66	0	105	Toxic death		11.2 ± 2.0	− 35	− 0.33
CDDP only	0	4.5	0	1849 ± 703	0.85	19.2 ± 3.3	11	
	0	9	0	1307 ± 790**	0.60	20.2 ± 4.5*	17	
	0	18	0	941 ± 323**	0.43	25.5 ± 4.3	47	
CDDP + 5-FU	0	4.5	105	826 ± 315**	0.38	20.6 ± 2.9	19	0.51
	0	9	105	629 ± 214**	0.29	25.7 ± 3.1**	49	1.14‖
	0	18	105	142 ± 137**	0.07	13.7 ± 2.7	− 21	− 0.29

*$P < 0.05$; †i.v.×3 (days 3, 7, 11); ‡i.v.×3 (day 3, 7, 11); §i.v.×3 (days 3, 7, 11); ¶on day 11; **$P < 0.01$ for no treatment by Bonferroni's test; ‖, Synergistic effect; S.D., standard deviation; T/C, treated/control ratio; ILS, increased life span; C.I., combination index.

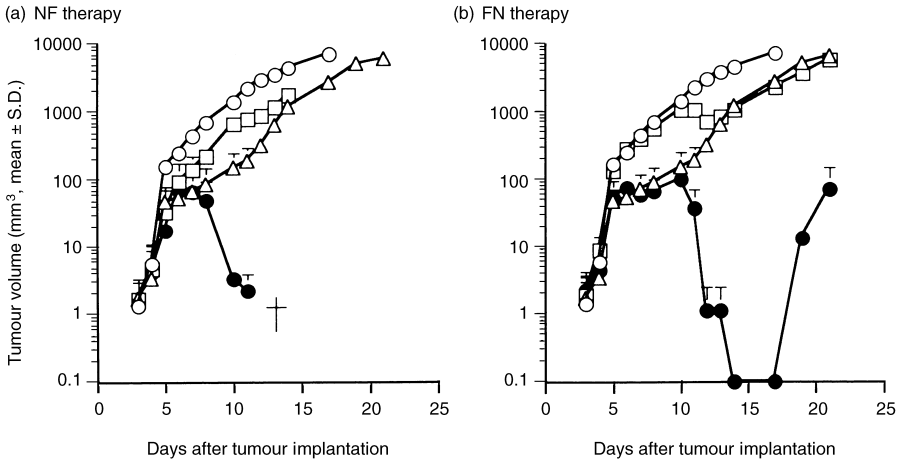

Figure 2. Growth inhibition of Lewis lung carcinoma cells by combination chemotherapy of nedaplatin (NDP) with 5-fluorouracil (5-FU). Total doses of NDP and 5-FU were 44 and 65.5 mg/kg, respectively. Mice bearing Lewis lung carcinoma were treated with saline (○), 5-FU (△), NDP (□) or the combination (●) in NF therapy (a) or FN therapy (b). †All animals died.

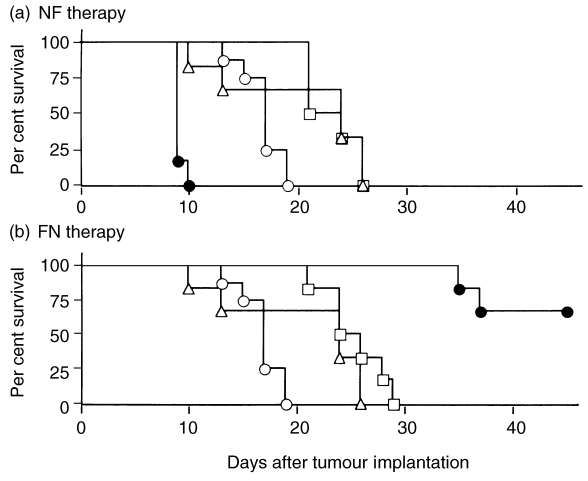

Figure 3. Prolonged survival after combination chemotherapy of nedaplatin (NDP) with 5-fluorouracil (5-FU). The experimental protocol was the same as in Figure 1. Mice bearing Lewis lung carcinoma were treated with saline (○), 5-FU (△), NDP (□) or the combination (●) in NF therapy (a) or FN therapy (b).

NF and FN therapy against Lewis lung carcinoma

It seems clear that the sequential administration of 5-FU followed by NDP showed more potent antitumour efficacy and less cytotoxicity than the reverse sequence. To confirm this sequence-dependent efficacy, a comparative study of NF versus FN therapy was performed. As shown in Figure 2, although regression of the tumours was observed in both NF and FN therapy at the 44 mg/kg dose, all the mice died during the course of the NF therapy. In contrast, no mice died during the FN therapy and four of six mice survived as tumour-free for more than 45 days (Figure 3). Even at 22 mg/kg of NDP, one of six mice was cured with the FN therapy (data not shown). Thus, the sequential dependency of antitumour efficacy was again clearly observed as a survival effect.

Figure 4 demonstrates the body weight change during NF and FN therapy. The body weight loss in the use of either drug alone was tolerable. While enhanced body weight loss was detected with the maximum loss of 15% of initial weight on day 12 in the FN therapy, body weight recovered thereafter. In contrast, the body weight of mice given NF therapy continuously decreased during the therapy, followed by toxic death.

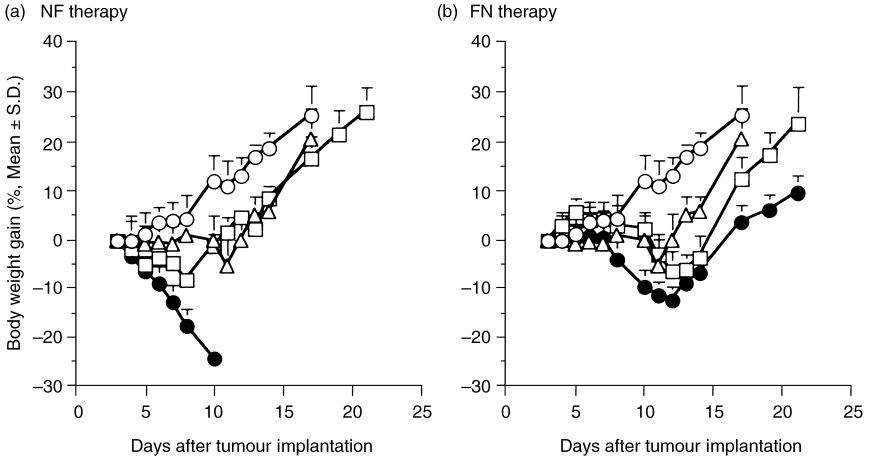

Figure 4. Body weight change during combination chemotherapy of nedaplatin (NDP) with 5-fluorouracil (5-FU). The experimental protocol was the same as in Figure 1. Body weight was measured and compared with the initial body weight. Mice bearing Lewis lung carcinoma were treated with saline (○), 5-FU (△), NDP (□) or the combination (●) in NF therapy (a) or FN therapy (b).

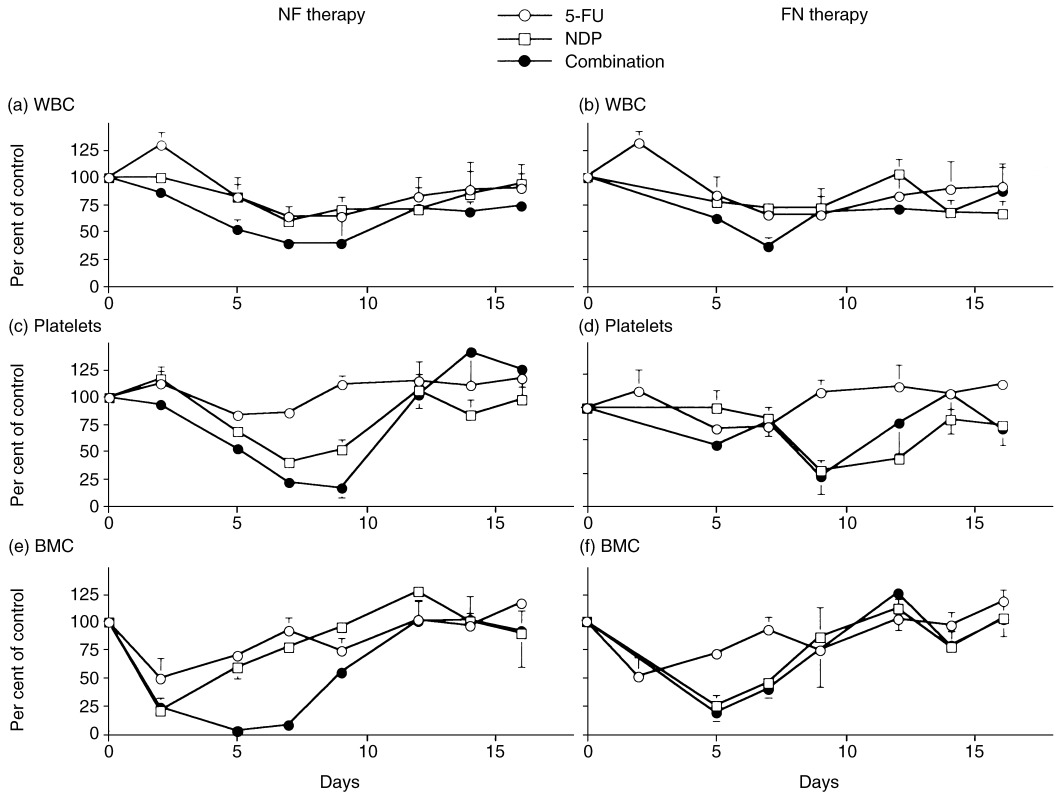

Figure 5. Haematotoxicity during combined treatment of nedaplatin (NDP) with 5-fluorouracil (5-FU) in NF and FN therapy. Doses of NDP and 5-FU used were 44 and 65.5 mg/kg, respectively. BMC, bone marrow cells.

The haematotoxicity of NF and FN therapy was analysed. To minimise the physiological influence of the growing tumour, non-tumour bearing BDF1 mice were used. After finishing the treatment, blood samples were collected and blood cells were counted. In FN therapy, no significant difference between the single and the combined treatment was detected in platelet and BMC counts (Figure 5d and f), whilst all parameters had a greater transient decrease with NDP and 5-FU alone compared with NF therapy (Figure 5c and e).

These results clearly demonstrate the sequence-dependent antitumour efficacy and toxicity of the combination of NDP with 5-FU. FN therapy, that is the administration of 5-FU followed by NDP, exerted potent antitumour efficacy without increased toxicity in this experimental therapeutic model.

DISCUSSION

Extensive preclinical and clinical trials of anticancer agents have been conducted using various administration schedules, in order to increase their antitumour efficacy. Although many studies have demonstrated that the combination therapy of CDDP and 5-FU works synergistically in various treatment schedules [11–16], the standard regimen for this combination still remains to be determined. In the case of NDP and 5-FU, in order to obtain the maximum antitumour effect in combination chemotherapy, the administration sequence of the drugs needs to be considered.

We examined three sets of administration schedules; 5-FU prior to NDP (FN therapy), 5-FU after NDP (NF therapy) and simultaneous intermittent administration (F + N therapy). The superior antitumour activity of FN therapy to other therapies was demonstrated in this study by an improvement in the length of survival. The FN therapy showed improved outcome with some mice becoming disease-free, while the NF therapy ended with toxic death of mice at the high-dose of NDP. The intermittent administration of NDP, which gave the maximum antitumour activity in the experimental study [20], did exert an inferior antitumour activity and increased toxicity at a high-dose of NDP in combination with 5-FU. Since survival is an important parameter for antitumour efficacy even in an experimental model, we believe this result to be a remarkable finding.

In order to clarify the cause of death at high-doses of NDP in the NF therapy, we first compared the profile of body weight changes in both NF and FN therapies. As shown in Figure 4, the maximum body weight losses and the day of nadir of mice by 5-FU alone, NDP alone in NF therapy and the NDP alone in FN therapy were 5.5% of initial body weight (day 11), 8.1% (day 8) and 6.4% (day 12), respectively. In spite of the fact that the days of nadir of the two drugs are closer in NF therapy, the profile of body weight changes is quite different between NF and FN therapy. These results suggest that the simple addition of body weight loss in monotherapy may not explain the increased toxicity in NF therapies. However, haematotoxicity analyses showed that the number of BMC decreased significantly with NF therapy but not with FN therapy (Figure 5). Thus, this haematotoxicity may explain the lethal toxicity in NF therapy and needs to be confirmed in the clinical study.

We also compared the antitumour activity of NDP plus 5-FU with that of CDDP plus 5-FU in all administration schedules. The sequence-dependent antitumour and toxic effect with the combination therapy of CDDP with 5-FU was similar to that of NDP with 5-FU. Although the increase in toxicity with the combination of CDDP with 5-FU has been

reported in clinical studies [23, 24], it is still not clear whether or not this enhanced toxicity is sequentially dependent.

We showed that antitumour efficacy was significantly higher with the combination of NDP plus 5-FU than with that of CDDP plus 5-FU, particularly in terms of survival. None of the mice given the combination of CDDP and 5-FU at any dose of any protocol used in this study had long-term tumour-free survival. In contrast, as described above, the sequence of 5-FU followed by NDP resulted in several long-term survivors.

The cell killing action of platinum complexes depends on the area under the curve (AUC) [25]. It has also been known that human plasma AUC of NDP and CDDP at each clinical dose, 100 and 80 mg/m^2, is 24.8 and 5.35 mg h/ml, respectively, the ratio of NDP/CDDP being 4.6 [26]. This ratio is greater than that from the animal study (the AUC ratio of NDP/CDDP was 3.7). These pharmacokinetical data suggest that the results of this study would be prospective in the clinical study.

Scanlon and colleagues [27] and Shirasaka and associates [12] demonstrated that CDDP inhibited methionine uptake into tumour cells, perturbed folate metabolism and made tumour cells more sensitive to 5-FU. This may explain the mechanism of the synergistic interaction of the two drugs in the sequence of CDDP followed by 5-FU, but not the reverse sequence. As to other mechanisms, it has been demonstrated that 5-FU inhibits the repair of CDDP-induced DNA damage [28] as well as glutathione synthesis which is responsible for the detoxification of CDDP in tumour cells [28, 29]. A precise understanding of the mechanisms of the synergistic interaction of NDP and 5-FU will be important for the appropriate use of these drugs in clinical studies. More extensive studies will be required to clarify these mechanisms.

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