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Combination Chemotherapy with Nedaplatin and Cyclophosphamide in Human Ovarian Cancer Model

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The antitumor efficacy of the combination of nedaplatin (NDP) with cyclophosphamide (CPM) was evaluated using human ovarian cancer models. Since NDP has been found to have greater antitumor activity and lower nephrotoxicity than cisplatin (CDDP), we also compared the antitumor activity of NDP plus CPM with that of CDDP plus CPM. Increasing doses of NDP (16.5, 33 and 66 mg/kg as a total dose) and a fixed amount of CPM (174 or 348 mg/kg as a total dose) were injected three times at intervals of 7 days via the tail vein into mice implanted with RMUG-S, OC9-JCK or KF-28 human ovarian cancer. Simultaneous administration of NDP with CPM resulted in markedly enhanced inhibition of tumor growth for all cancers tested. The growth inhibition and survival effect of the combination therapy of NDP with CPM against KF-28 and OC9-JCK were as potent as those of CDDP plus CPM. Neither increased hematotoxicity nor a significant difference in maximum concentration, half time or area under the curve of platinum or CPM in plasma between the single and combined treatment was found. These results suggest that the combination of NDP with CPM may be clinically effective.

Key words: Nedaplatin — Cisplatin — Cyclophosphamide — Combination chemotherapy — Ovarian cancer

Nedaplatin (NDP) was selected from a series of platinum analogs because of its pronounced antitumor activity against solid tumors including colon carcinoma, lung carcinoma, melanoma and breast cancer, with lower nephrotoxicity than cisplatin (CDDP), in preclinical studies.[1–5] In clinical Phase II studies, NDP showed good efficacy against lung,[6,7] head and neck,[8] testicular,[9] and gynecological[10] cancers. In previous studies, we have demonstrated that NDP shows synergistic antitumor activity against various cancers in combination with etoposide (ETP) or 5-fluorouracil (5-FU).[11,12]

Administration of a platinum complex plus an alkylating agent is standard therapy for advanced epithelial ovarian cancer[13–16] and the most commonly used combination is CDDP and cyclophosphamide (CPM).[17–19] Currently, the combination of CDDP and paclitaxel represents the new standard.[20] We have previously demonstrated that NDP showed synergistic antitumor efficacy in combination with CPM against murine and human lung cancer.[21] In the present study, we evaluated the augmentation of *in vivo* antitumor efficacy and toxicity in the combination chemotherapy of NDP plus CPM against three human ovarian cancers, including pharmacokinetic studies. We also compared the therapeutic efficacy of NDP plus CPM with that of CDDP plus CPM.

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MATERIALS AND METHODS

Animals BDF1 and athymic BALB/c nude mice (female, 7–9 weeks old) were purchased from Japan SLC Inc. (Shizuoka) and CLEA Japan Inc. (Tokyo), respectively.

Tumors OC9-JCK (human ovarian cancer) was provided by the Central Institute for Experimental Animals (Kawasaki). Human ovarian cancers RMUG-S and KF-28 were kindly provided by Dr. S. Nozawa (Keio University, Tokyo) and Dr. Y. Kikuchi (National Defense Medical College, Saitama),[22] respectively. All tumor cell lines were routinely maintained by serial s.c. transplantation as tumor fragments in BALB/c nude mice. RMUG-S and KF-28 were also maintained by *in vitro* passage using Eagle's MEM (Nissui Pharmaceutical Co., Tokyo) supplemented with 10% fetal calf serum (Life Technologies Inc., Rockville, MD).

Drugs NDP and CPM were obtained from Shionogi & Co., Ltd. (Osaka). CDDP was purchased from Nippon Kayaku (Tokyo). All drugs were dissolved in saline immediately before use.

***In vivo* therapeutic experiments** The experimental procedure was described previously.[11,12] In all experiments, 5 to 10 mice per group were used. On day 0, a tumor fragment (approximately 8 mm^3) was implanted s.c. into the back of BALB/c nude mice. Treatment was started when the tumor volume reached approximately 100 mm^3. All drugs were administered i.v. once a week for three weeks.

The total doses of drugs used were 16.5, 33 and 66 mg/kg for NDP, 9 mg/kg for CDDP, and 174 or 384 mg/kg for CPM. The maximum tolerated doses (MTDs) of NDP, CDDP and CPM in the route and schedule used in this study were 66, 18 and 348 mg/kg, respectively. The MTDs in combination therapy are either 66 mg/kg of NDP (or 18 mg/kg of CDDP) plus 174 mg/kg of CPM or 33 mg/kg of NDP (or 9 mg/kg of CDDP) plus 348 mg/kg of CPM, because combined treatment with 66 mg/kg of NDP and 348 mg/kg of CPM resulted in toxic death or severe body weight losses. All experiments were performed with the approval of the Shionogi Animal Care and Use Committee.

Evaluation of antitumor efficacy Tumor size, body weight and survival were scored throughout each experiment. The endpoint for survival was taken as the time when animals became moribund, exhibiting hypoactivity or hypothermia. The mice were then killed. Relative growth inhibition (RV) was calculated as $RV=V_n/V_0$, where V_n: tumor volume on day n, and V_0: initial tumor volume. The growth-inhibitory effect and prolongation of survival were estimated in terms of the treated/control ratio (T/C) and increased life span (ILS%) versus the control, respectively. For the evaluation of combination therapies, the combination index (CI) was used.

The CI was calculated from ILS% values as follows[11]: CI=ILS% of drug A with drug B/(ILS% of drug A+ILS% of drug B).

A CI of more than 1 indicates synergy, a CI equal to 1 indicates additivity and a CI of less than 1 indicates antagonism.

Statistics In this study, the statistical significance of differences between treated and non-treated groups or between treated groups was evaluated using Bonferroni's test and Dunnett's test, respectively.[23, 24]

Hematotoxicity study NDP (22 mg/kg) and/or CPM (116 mg/kg) were i.v. injected into BDF1 mice and blood samples were collected from the portal vein of anesthe-

Table I. Augmentation of Antitumor Activity in Combination Chemotherapy of Nedaplatin (NDP) with Cyclophosphamide (CPM) against Human Ovarian Cancer

Cancer	Group	Total dose (mg/kg)		RV[b] (Mean±SD)	T/C[c]	Maximum body weight loss (%)[d]
		NDP[a]	CPM[a]			
KF-28	Untreated control	0	0	33.4±11.3		0.0
	CPM only	0	87	14.3±3.2**	0.43	3.5
		0	174	4.4±1.9**	0.13	7.3
		0	348	1.9±1.0**	0.06	9.0
	NDP only	33	0	17.0±0.6**	0.51	2.8
		66	0	4.2±2.1**	0.13	15.1
	Combination	33	174	1.1±0.9**	0.03	9.3
		66	174	0.02±0.01**	0.00	17.2
OC9-JCK	Untreated control	0	0	15.2±9.2		0.0
	CPM only	0	174	3.0±1.5**	0.20	8.0
		0	348	1.7±0.6**	0.11	6.2
	NDP only	16.5	0	7.9±3.5**	0.52	8.2
		33	0	2.8±1.9**	0.18	9.1
	Combination	16.5	348	1.1±0.5**	0.07	4.5
		33	348	0.6±0.3**	0.04	10.0
RMUG-S	Untreated control	0	0	7.8±2.6		5.7
	CPM only	0	174	7.7±2.4	0.99	7.7
		0	348	4.8±1.5	0.62	7.7
	NDP only	16.5	0	7.2±2.4	0.92	3.1
		33	0	8.1±2.4	1.04	6.3
	Combination	16.5	348	4.8±1.3	0.62	11.3
		33	348	3.8±1.2*	0.49	10.1

a) i.v. ×3 (days 15, 22, 29 for RMUG-S, days 21, 28, 35 for OC9-JCK, days 12, 19, 26 for KF-28).
b) Relative tumor volume on day 36 for RMUG-S, on day 42 for OC9-JCK, on day 62 for KF-28.
c) Treated/control.
d) % of initial.
*, ** $P<0.05$, 0.01 for no treatment by Bonferroni's test.

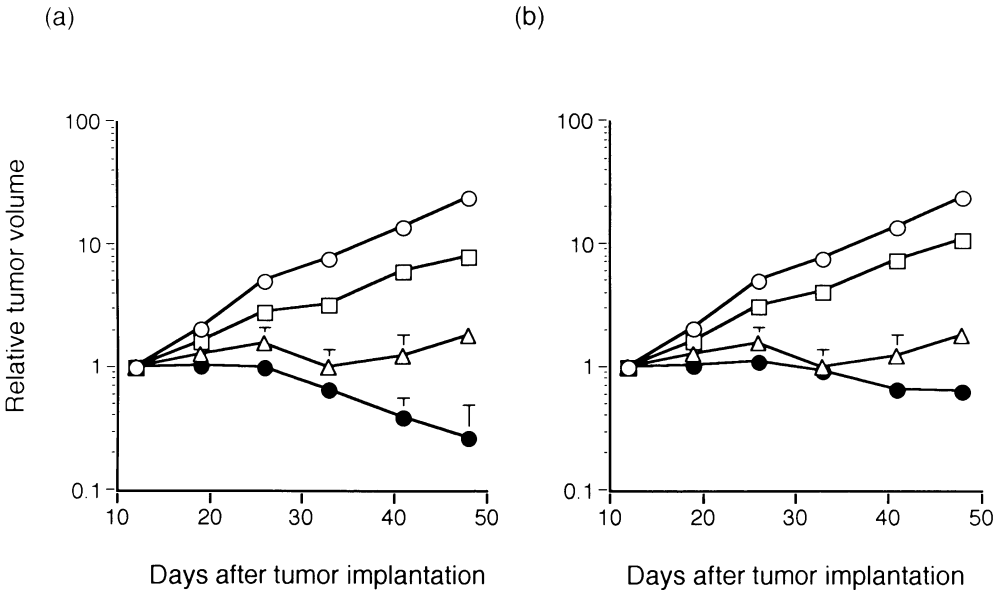

Fig. 1. Augmented growth inhibition of KF-28 human ovarian cancer by combination chemotherapy with nedaplatin (NDP) plus cyclophosphamide (CPM) (a) and cisplatin (CDDP) plus CPM (b). The experimental protocols are shown in "Materials and Methods." NDP (11 mg/kg), CDDP (3 mg/kg) and CPM (58 mg/kg) were injected i.v. on days 12, 19 and 26. Mice were treated with saline (○), CPM (△), NDP or CDDP (□) or a combination (●). Mean relative tumor volume±SD were shown.

tized mice on 1, 3, 5, 7, 9, 11, 14 and 17 days after the treatment. Nucleated bone marrow cells were collected from the right femur. The numbers of white blood cells, red blood cells, platelets and bone marrow cells were counted with an automatic cell counter (Sysmex K-1000 and CDA-500, Kobe).

Pharmacokinetic study NDP (22 mg/kg) and/or CPM (116 mg/kg) were injected i.v. into BDF1 mice and blood samples were harvested with a heparinized syringe at 5, 30, 60, 120, 240 and 480 min, followed by rapid centrifugation at 600*g* for 5 min to separate plasma. The platinum content of plasma was determined with a flameless atomic absorption spectrometer (model Hitachi Z-5000, Tokyo). With regard to CPM, the concentration of 4-hydroxycyclophosphamide (4-HCP), an active metabolite of CPM, was determined by means of the fluorometric method as described previously.[25]

***In vitro* cell culture** For determination of the *in vitro* cytotoxic activity of NDP and CPM, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used as described previously.[26] KF-28 cells (3000 cells/well) were plated in a 96-well culture plate (Sumitomo Bakelite Co., Tokyo) in 100 μl of culture medium. Twenty-four hours later, serial dilutions of NDP and/or 4-HCP (100 μl) were added to the wells. After a 24 h incubation, the culture medium was removed and the wells were washed three times with Eagle's MEM followed by additional culture in a CO_2 incubator (Tabai Espec Corp., Osaka) for 4 days and cytotoxicity was determined by means of MTT assay. The IC_{50} values of NDP and CPM for KF-28 cells under the above experimental conditions were 0.9 and 1.1 μM, respectively.

RESULTS

Combination therapy of NDP plus CPM against human ovarian cancer Table I summarizes the results of combination therapy with NDP and CPM against three human ovarian cancers. In all human ovarian cancer models tested, combination therapy of NDP with CPM resulted in enhanced antitumor efficacy. This combination showed greater growth-inhibitory activity than NDP or CPM alone in both KF-28 and OC9-JCK cells. RMUG-S cells, which were rather less sensitive to continuous exposure to NDP *in vitro* for 4 days (IC_{50}=6.3 μM, data not shown), did not respond to NDP in this experimental therapy. However, tumor growth was significantly (P<0.05) and effectively (T/C<0.5) inhibited at a high dose of NDP in combination with CPM. Although body weight loss during therapy was augmented by the combination of NDP with CPM, it remained within a tolerable range (<20% of the initial body weight) as shown in Table I.

Combination therapy with NDP plus CPM versus CDDP plus CPM against human ovarian cancer Fig. 1

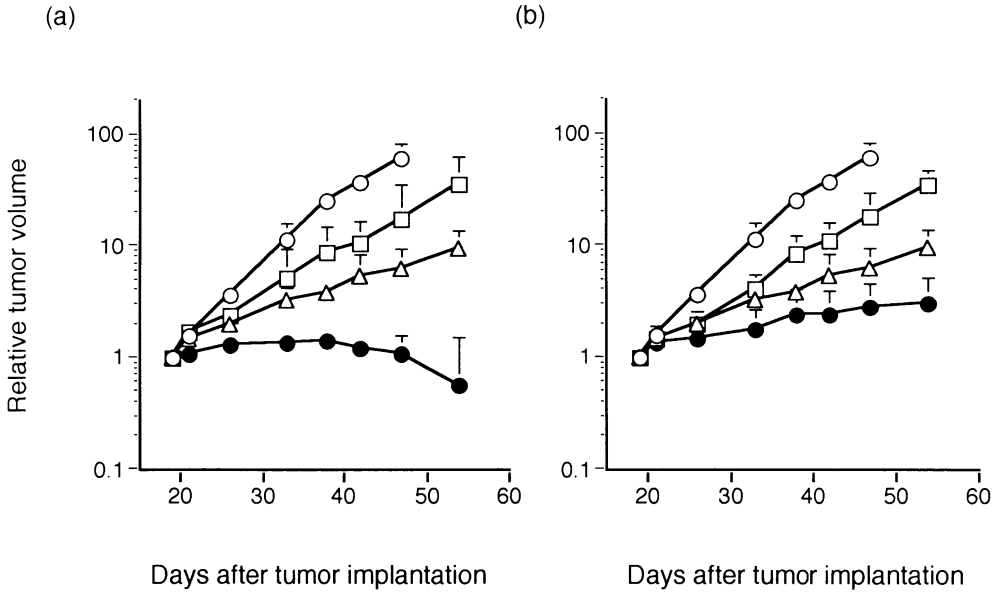

Fig. 2. Augmented growth inhibition of OC9-JCK human ovarian cancer by combination chemotherapy with nedaplatin (NDP) plus cyclophosphamide (CPM) (a) and cisplatin (CDDP) plus CPM (b). The experimental protocols are described in "Materials and Methods." NDP (11 mg/kg), CDDP (3 mg/kg) and CPM (58 mg/kg) were injected i.v. on days 19, 26 and 39. Mice were treated with saline (○), CPM (△), NDP or CDDP (□) or a combination (●). Mean relative tumor volume±SD were shown.

Table II. Prolonged Survival of OC9-JCK-bearing Mice after Combination Chemotherapy with Nedaplatin (NDP) and Cyclophosphamide (CPM) and Cisplatin (CDDP) with CPM

Group	Total dose (mg/kg)		Survival days (individual mice)	MSD[b)]	CI[c)]
	NDP[a)]	CPM[a)]			
Untreated control	0	0	68, 58, 66, 70, 61, 58, 54, 62	62.1±5.6	
CPM only	0	174	82, 89, 90, 91, 81, 80	85.5±5.1**	
NDP only	33	0	75, 79, 80, 77, 85, 58	75.7±9.5	
Combination	33	174	116, 110, 107, >150, 114, 100	>116.2±17.9**	>1.45
CDDP only	9	0	72, 68, 72, 82, 75, 84	75.5±6.4	
Combination	9	174	103, 89, 110, 89, 121, 104	102.7±12.6**	1.08

a) i.v. × 3 (days 21, 28, 35).
b) Mean survival days (mean±SD).
c) Combination index.
** $P<0.01$ for no treatment by Bonferroni's test.

shows the results obtained with NDP plus CPM and CDDP plus CPM (33 mg/kg NDP and 9 mg/kg CDDP) against KF-28 human ovarian cancer. While growth inhibition was enhanced by combined treatment in both cases, the growth-inhibitory activity of NDP plus CPM was superior to that of CDDP plus CPM. A similar but less marked result was obtained with OC9-JCK cells (Fig. 2). Table II shows the effect of both therapies on the survival of OC9-JCK-inoculated mice. The survival time was significantly ($P<0.05$) increased when mice were treated with NDP and CPM in comparison with either NDP or CPM alone. The combination of CDDP with CPM also resulted in prolonged survival. The mean survival periods of mice treated with NDP plus CPM and CDDP plus CPM were 116±18 and 103±13 days, respectively, indicating comparable survival effects for both treatments. The CI values indicated that the effect was synergistic in the case of NDP plus CPM, but nearly additive in the case of CDDP plus CPM (CI values for NDP plus CPM and CDDP plus CPM were 1.45 and 1.08, respectively). One of six OC9-JCK-bearing mice treated with NDP and CPM survived more than 150 days without recurrence of the tumor.

Table III. Hematotoxicity of Combined Treatment of NDP with CPM

Group[b)]	Maximum reduction[a)] (day of nadir)			
	White blood cells	Red blood cells	Platelets	Bone marrow cells
Untreated control	100±3.5[c)]	100±1.9[c)]	100±3.5[c)]	100±2.0[c)]
CPM only	18±1.0** (3)	75±3.3** (3)	86±18.7 (3)	23±5.0** (3)
NDP only	59±4.1** (14)	86±5.6* (7)	41±2.0** (9)	68±1.2** (5)
Combination	18±1.2** (3)	74±0.2** (7)	63±6.6** (7)	14±2.2** (3)

NDP or CPM was injected i.v. on day 0. Doses of NDP and CPM used were 22 mg/kg and 116 mg/kg, respectively.
a) Percent of untreated control, mean±SD.
b) n=3.
c) Ranges of the mean cell numbers of white blood cells, red blood cells, platelets and bone marrow cells at the various time points were $3.8–5.7\times10^3/\mu$l, $7.2–7.9\times10^6/\mu$l, $7.6–8.6\times10^5$/ml and $1.1–1.5\times10^7$/femur, respectively.
*, ** $P<0.05$, 0.01 for untreated control by Bonferroni's test.

Toxicity study The hematotoxicity of NDP with CPM was analyzed (Table III). To minimize the physiological influence of the growing tumor, non-tumor-bearing BDF1 mice were used for this study. NDP (22 mg/kg) and/or CPM (116 mg/kg) were injected i.v. and blood samples were collected 1, 3, 5, 7, 9, 11, 14 and 17 days thereafter. No significant augmentation of hematotoxicity was detected with the combination of NDP and CPM in terms of any parameter. These results demonstrated that combined chemotherapy with NDP and CPM exhibited potent antitumor efficacy against human ovarian cancer without increased toxicity.

***In vitro* cytotoxicity study** In order to clarify the mechanism of the enhancement of antitumor efficacy, *in vitro* cytotoxicity experiments were done by means of MTT assay using KF-28 cells. One-day exposure to NDP and 4-HCP, an active form of CPM, resulted in additive cytotoxic activity at a low dose (0.2 μM NDP and 0.6 μM CPM), but antagonistic activity at a high dose (0.8 μM NDP and 2.4 μM CPM) (Fig. 3).

Pharmacokinetic study The pharmacokinetics of NDP and CPM were analyzed. No significant difference between the single and the combined treatment was found in terms of the maximum concentration (C_{max}), half time ($t_{1/2}$) and area under the curve (AUC) of platinum or CPM in plasma (Table IV).

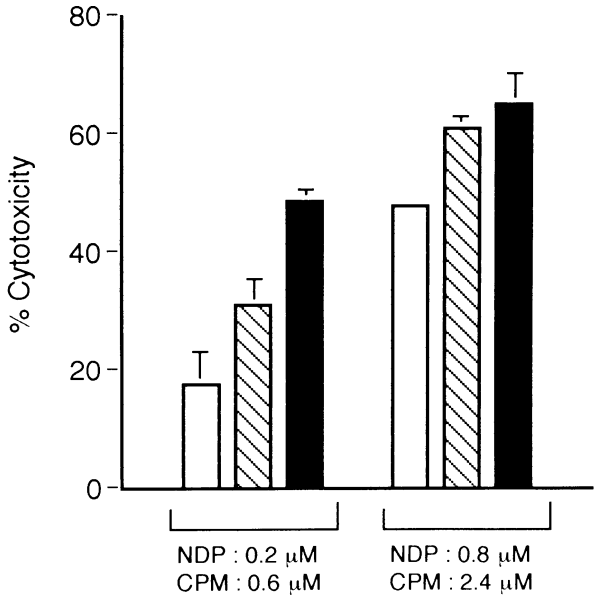

Fig. 3. *In vitro* cytotoxic activity of NDP plus CPM combination against KF-28 human ovarian cancer. Cytotoxic activity was determined by MTT assay as described in "Materials and Methods." NDP (open column), 4-HCP (hatched column), combination (solid column).

DISCUSSION

Extensive preclinical and clinical trials of anticancer agents in various combinations have been conducted in order to increase antitumor efficacy. For advanced ovarian cancer, randomized trials comparing clinical response rates and progression-free survival have established that CDDP-based combination therapy is more effective than single-agent CDDP, single-agent alkylating agents, or combinations without CDDP.[13–19, 27, 28)]

We have previously demonstrated the antitumor efficacy of NDP with CPM against murine and human lung can-

Table IV. Pharmacokinetics of NDP and CPM

Group	Platinum			CPM		
	C_{max} a)	$t_{1/2}$ b)	AUC_{0-8} c)	C_{max} a)	$t_{1/2}$ b)	AUC_{0-1} c)
CPM only	ND	ND	ND	165.9	0.47	130.7
NDP only	170.2	0.17	59.6	ND	ND	ND
Combination	166.8	0.18	59.8	133.1	0.50	115.3

NDP (22 mg/kg) and/or CPM (116 mg/kg) were i.v. injected.
a) μM.
b) Hour.
c) $\mu M \times$h.
ND: not done.

cer.[21] In the present study, we focused on the antitumor efficacy of this therapy against human ovarian cancer. In order to examine effective responses against human ovarian cancers used in this study, which are slow-growing in nude mice, NDP and CPM were administered 3 times with 7-day intervals. Our preliminary data demonstrated that the repeated administration of NDP and CPM was more effective than single treatment (data not shown). Enhancement of growth inhibition (Table I, Fig. 1 and Fig. 2) in NDP plus CPM therapy was demonstrated against three human ovarian cancers tested, including the NDP-sensitive tumor KF-28 and the insensitive tumor RMUG-S. In the OC9-JCK model, prolonged survival was also afforded by the combination of NDP with CPM (Table II). The antitumor efficacy of NDP with CPM was superior to the maximum response to either NDP or CPM monotherapy.

We also compared the antitumor activity of NDP plus CPM with that of CDDP plus CPM against KF-28 and OC9-JCK tumors. In the KF-28 model, it was clear that NDP plus CPM was superior to CDDP plus CPM in terms of tumor growth inhibition. Although similar results were also obtained with the OC9-JCK tumor, the T/C values on day 54 in NDP plus CPM and CDDP plus CPM were 0.56 ± 0.68 and 3.00 ± 2.14, respectively, and the difference was not statistically significant (Fig. 2). However, regression of the tumors was found in five of six mice treated with NDP plus CPM even 15 days after the last treatment, but in only one of six mice given CDDP plus CPM therapy. In the experiment using KF-28 cells, tumor regression was also observed in six of six mice given NDP with CPM and in two of six mice given CDDP with CPM (Fig. 1). These results indicated that the combination of NDP with CPM is more effective than that of CDDP with CPM in terms of response rate. It is also noteworthy that three of five KF-28-bearing mice (Fig. 1, data not shown) and one of six OC9-JCK-bearing mice (Table III) treated with NDP and CPM survived more than 150 days without tumor recurrence. No mouse treated with CDDP plus CPM was found to be cured. These results suggest that the combination of NDP plus CPM confers a survival benefit and confirmed our previous finding with murine Lewis lung carcinoma.[21]

Toxicity, in addition to efficacy, must be considered in combination chemotherapy. We therefore first compared the profile of body weight changes among the therapies. As shown in Table I, the maximum body weight losses in animals given NDP plus CPM therapy were within the tolerable range (<20% of the initial body weight) on the administration schedule used in this study. We therefore evaluated the hematotoxicity in the combination therapy at 22 mg/kg of NDP and 116 mg/kg of CPM as a single injection. The numbers of white blood cells, red blood cells and bone marrow cells were mainly decreased by CPM treatment and the number of platelets was mainly decreased by NDP treatment. However, hematotoxicity was not augmented by the combination of the two agents. Thrombocytopenia, a dose-limiting factor of NDP, was not enhanced by the combination with CPM. This should be beneficial for the combination chemotherapy of NDP with CPM, and awaits confirmation in clinical trials.

CDDP/NDP and CPM damage DNA by two different mechanisms; the involvements of DNA strand "kinking" and DNA strand "cross-linking" may explain the positive cell killing interaction between these two drugs.[29] Thus, we first performed *in vitro* cytotoxicity experiments with KF-28 cells using MTT assay in order to investigate the mechanism of the interaction of NDP and CPM found in the *in vivo* study. Simple *in vitro* exposure to NDP and CPM for 1 day showed additive cytotoxic activity at a low dose but antagonistic activity at a high dose (Fig. 3). A similar result was obtained in the 4-day exposure experiment in which the total AUCs of NDP and CPM were greater than those used in *in vivo* therapeutic experiments (data not shown). These results suggest that the cell killing interaction may not be sufficient to explain the enhanced *in vivo* antitumor efficacy of the combination therapy.

We next examined the pharmacokinetics of NDP and CPM administered alone or in combination. The pharmacokinetic parameters, such as C_{max}, $t_{1/2}$ and AUC of NDP or CPM, were not affected by the simultaneous adminis-

tration. This finding was supported by the results of an earlier clinical pharmacokinetic study.[30] The enhanced antitumor activity, therefore, may not be due to the altered pharmacokinetics of NDP or CPM, although other possibilities, for example higher intra-tumor drug concentration or delayed clearance from tumor tissue upon simultaneous administration of the two drugs, remain to be checked. A precise understanding of the mechanism of the interaction of NDP and CPM is important for appropriate clinical use of these drugs.

In conclusion, the results presented in this study suggest the potential efficacy of a combination of NDP with CPM for clinical therapy.

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