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Growth-inhibitory Effects of *N,N*-Diethyl-2-[4-(phenylmethyl)phenoxy]-ethanamine-HCl Combined with Cisplatin on Human Ovarian Cancer Cells Inoculated into Nude Mice

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In 5-day incubation of an estrogen receptor-negative human ovarian cancer cell line (KF) with *N,N*-diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine-HCl (DPPE), the concentration of DPPE required for 50% inhibition of KF cell proliferation (IC_{50}) was 1.7 μM. The IC_{50} of DPPE for inhibition of protein kinase C (PKC) activity was 3.0 μM, a similar value to those of other antiestrogens such as tamoxifen and clomiphene. DPPE also inhibited phosphorylation of mitogen-activated protein kinase in KF cells. When treatment with DPPE was started 7 days after inoculation of KF cells into nude mice, 50 mg/kg DPPE alone resulted in a significant growth retardation in the early stage of tumor growth. Although 25 mg/kg DPPE showed a similar effect to 2 mg/kg cisplatin (CDDP), the combination had the most marked tumor growth-inhibitory effect. Nude mice treated with combinations of CDDP and DPPE survived significantly longer than not only untreated, but also CDDP-alone-treated mice, while 50 mg/kg but not 25 mg/kg DPPE alone had an effect comparable to that of 2 mg/kg CDDP alone. If treatment with DPPE was begun from the day after tumor inoculation, the inhibitory effect of DPPE was further enhanced, especially when combined with CDDP. If treatment with DPPE was started in nude mice with a lower tumor burden, 25 mg/kg as well as 50 mg/kg DPPE had a similar effect to 2 mg/kg CDDP, in terms of survival. When DPPE was combined with CDDP, the effect was significantly enhanced, compared to that of either alone. These treatments could be done without any adverse side effect. Thus, we conclude that DPPE has an antiestrogen action and its tumor growth-inhibiting activity is enhanced on administration in combination with CDDP.

Key words: DPPE — Cisplatin — Ovarian cancer cells — Nude mice

The paradiphenylmethane tamoxifen (TAM) derivative, *N,N*-diethyl-2-[4-(phenylmethyl)phenoxy]ethanamine-HCl (DPPE) was synthesized by Brandes and Hermonat.[1)] Kikuchi *et al.*[2)] previously reported that antiestrogens such as clomiphene and TAM had a potent antiproliferative effect on estrogen receptor-negative ovarian cancer cells and could potentiate the antiproliferative effect of cisplatin (CDDP). Like TAM, a diphenylmethane-type antiestrogen binding site (AEBS) ligand, DPPE is antiproliferative *in vitro* and binds with high affinity to AEBS.[3)] In addition, we have found that when the CDDP sensitivity was reduced by treatment with 12-*O*-tetradecanoylphorbol-13-acetate, the cellular protein kinase C (PKC) rose, and when the CDDP sensitivity was increased, the cellular PKC decreased, suggesting that the defense mechanisms of cells against cytotoxicity to CDDP are transduced through PKC.[4)] Mitogen-activated protein kinase (MAPK) (also referred to as extracellular signal-regulated kinase) is activated following growth factor stimulation through a signaling cascade involving Ras, Raf, and MAPK.[5)] Activated MAPK, in turn, plays a critical role in the activation of transcription factors involved in regulating proliferation-associated genes.[6,7)] Therefore, the effects of DPPE on the cellular PKC and MAPK activities were examined to address the roles of the PKC and MAPK signaling pathways in the inhibition of ovarian cancer cell proliferation *in vitro*.

DPPE promotes the growth of transformed or malignant cells with a bell-shaped dose-response curve. At low doses (1 to 4 mg/kg), DPPE accelerated tumor growth in rodents.[8)] As the dose of DPPE is increased, growth promotion decreases, and, at higher concentrations *in vitro*, DPPE is antiproliferative/cytotoxic to 40/40 murine or human cell lines tested in the National Cancer Institute screening panel. In the previous study, we demonstrated that 5 mg/kg DPPE alone did not significantly inhibit the tumor growth of nude mice bearing human ovarian cancer cells, while a combination of CDDP and 10 mg/kg DPPE caused a significant prolongation of the survival without significant antitumor responses,[9)] suggesting host-mediated effects. Brandes *et al.*[10)] revealed that higher concentrations of DPPE significantly potentiate the cytotoxicity of antineoplastic drugs to cancer cells and result in a high cure rate. The combined effect of

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DPPE and CDDP on the cell proliferation has been confirmed *in vitro*.[9] Based upon the *in vitro* results, we examined whether the combined effect of DPPE and CDDP can also be seen *in vivo* using a tumor-bearing nude mouse model.

Thus, in the present study we attempted to elucidate in further detail the mechanism of action of DPPE *in vitro* and the effects of higher concentrations of DPPE and their combination with CDDP on the tumor growth and survival of nude mice bearing human ovarian cancer cells.

MATERIALS AND METHODS

Chemicals CDDP was obtained from Bristol-Myers Squibb Co., Ltd. (Tokyo). DPPE was kindly supplied by Dr. Lorne J. Brandes. TAM and clomiphene were purchased from Sigma Chemical Company (St. Louis, MO). Chemical structures of DPPE, TAM and clomiphene are described in Fig. 1. These drugs were dissolved and diluted with medium or saline to the desired concentrations prior to use.

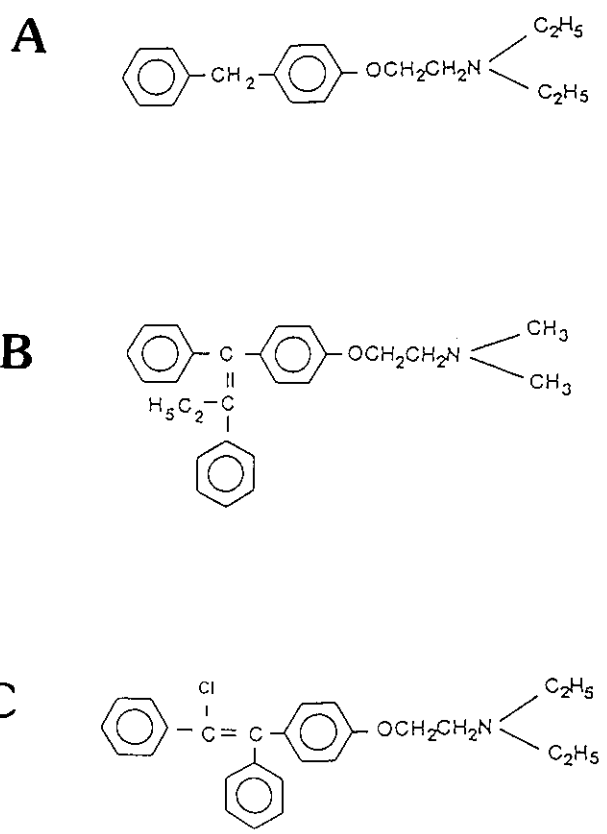

Fig. 1. Chemical structures of DPPE (A), tamoxifen (B) and clomiphene (C).

Cell culture Human ovarian cancer cell line (KF) cells were established from tissue of a patient with serous cystadenocarcinoma of the ovary.[11] This cell line was incubated in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 m*M* glutamine, 100 units of penicillin/ml and 100 μg of streptomycin/ml (GIBCO, Grand Island, NY) in 5% CO_2 at 37°C. The medium was changed every 3 days, and the cells were passaged when they reached confluence.

***In vitro* treatment** To determine the concentrations of CDDP, DPPE, TAM and clomiphene required for 50% inhibition of KF cell proliferation *in vitro* (IC_{50}), we seeded 5×10^2 KF cells in 96-well flat-bottomed microtest plates (Becton-Dickinson Co., Mountain View, CA) and incubated them in the absence or presence of drugs in a humidified atmosphere of 5% CO_2 at 37°C. After incubation for 5 days, cytotoxicity was evaluated using a crystal violet staining method.[12] Briefly, an equal volume of 10% formalin in phosphate-buffered saline containing 0.2% crystal violet was added to each well and after incubation at room temperature, the absorbance at 590 nm of stained cells in each well was measured with an automatic microtest-plate reader (Multiscan MCC/340, Titertek, Flow Laboratories Inc., VA). Average absorbance of the control wells in the absence of drugs was regarded as 100%, and the percentage cell growth in each well was calculated. The concentrations of drugs that inhibited the growth of cells to the level of 50% of the control growth (IC_{50}) was obtained from graphical plots. The viability was assessed by trypan blue dye exclusion.

Assay for PKC activity An aliquot of 10^7 cells was suspended in 1 ml of 50 m*M* Tris-HCl (pH 7.5) buffer containing 5 m*M* EDTA, 10 m*M* EGTA, 0.3% w/v β-mercaptoethanol, 10 m*M* benzamidine and 50 μg/ml phenylmethylsulfoxyl fluoride to prevent protein degradation. The cell suspension was sonicated and centrifuged at 105,000*g* for 1 h. The supernatants containing the cytosolic fraction of the enzyme were kept at 4°C until use. The cytosolic fractions were used for PKC assay. Enzyme activity was determined by means of a Protein Kinase C enzyme assay kit (Amersham, Aylesbury, UK). The assay used is a modification of a mixed micelle assay, with phorbol 12-myristate 13-acetate to activate the enzyme. One unit of PKC is defined as the amount of enzyme which catalyzes the transfer of 1 pmol of phosphate from ATP to histone per min at 30°C. Concentrations of drugs required for 50% inhibition of PKC (IC_{50}) were compared.

MAPK assay KF cells were seeded into 6-well plates, grown to confluency and then incubated in serum-free medium for 24 h. The cells were then incubated with RPMI 1640 medium containing 10% FCS or 10 μ*M* DPPE with 10% FCS for 10 min. The cell monolayers

were washed with fresh medium and harvested in hypotonic lysis buffer (25 m*M* HEPES, pH 7.4, 5 m*M* ethylenediaminetetraacetic acid, 1 m*M* phenylmethylsulfoxyl fluoride, 1 m*M* Na-orthovanadate, 10 μg/ml leupeptin). The cell extracts were clarified by centrifugation for 10 min at 12,000*g*. Western blotting was performed after electrophoretic transfer onto a polyacrylamide gel (SDS-PAGE), using mouse anti-MAPK (ERK 1+2) monoclonal antibody (clone Z033, Zymed Laboratory, South San Francisco, CA).

Nude mice Six-week-old female BALB/c nude mice were obtained from Japan Clea Laboratories, Tokyo, and maintained in a pathogen-free environment. The animals were inspected daily and tumor growth was determined with a caliper. When necessary, animals were killed and dissected. The tumor tissues were fixed in formalin for histological examination. The larger tumors (more than 2 cm in diameter) contained a larger necrotic area in the center. Distant metastases were not observed during the experimental period. Unless treated, all tumor-bearing mice died of tumor burden within 100 days of tumor inoculation.

***In vivo* treatment** To determine the combined effect of CDDP and DPPE on the tumor growth and the survival, 5×10^5 KF cells were inoculated s.c. into the right flank of nude mice. In the first protocol, treatments were started from 7 days after tumor inoculation. Treatment groups were divided as follows: group 1 ($n=10$), medium alone was administered i.p. once a week for 6 weeks; group 2 ($n=10$), 2 mg/kg CDDP alone was administered i.p. once a week for 6 weeks; group 3 ($n=10$), 25 mg/kg DPPE alone was administered i.p. once a week for 6 weeks; group 4 ($n=10$), 50 mg/kg DPPE alone was administered i.p. once a week for 6 weeks; group 5 ($n=10$), 2 mg/kg CDDP and 25 mg/kg DPPE were simultaneously administered i.p. once a week for 6 weeks; group 6 ($n=10$), 2 mg/kg CDDP and 50 mg/kg DPPE were simultaneously administered i.p. once a week for 6 weeks. In the second protocol, treatments were started from the day after tumor inoculation. Treatment groups were divided in the same way as in the first protocol. Each injection was given in a 0.15 ml volume. The tumor growth was determined by measurement of the diameters of the tumor nodule in two dimensions with a caliper once a week. Tumor volume (cm^3) was calculated according to the following formula: $4\pi/3\times(r_1+r_2)^3/8$, where r_1 is the longitudinal radius and r_2 is the transverse radius. Blood from a tail vein was collected into hematocrit tubes every week and the hematocrit values and body weight were recorded for monitoring the side effects of drugs. In these experiments, the observation period was 120 days.

Statistical analyses Where indicated, statistical significance was tested using the Mann-Whitney U test and the Cox Mantel test; a P value <0.05 was considered significant.

RESULTS

As shown in Table I, the IC_{50} values of DPPE and clomiphene were 1.7 and 1.9 μM, respectively, being about 4–5 fold higher than that of CDDP, while TAM showed about 10-fold higher IC_{50} than CDDP.

The cytosolic PKC activity in the KF cells was most markedly inhibited by DPPE ($IC_{50}=3.0$ μM), while TAM ($IC_{50}=5.5$ μM) and clomiphene ($IC_{50}=6.5$ μM) showed similar inhibitory effects (Table II). As shown in Fig. 2, the phosphorylation of MAPK induced by serum in KF cells was inhibited by DPPE (10 μM) as evaluated from the mobility shift on electrophoresis.

When treatment was performed from 7 days after tumor inoculation, the tumor growth curves in mice treated with CDDP alone and 25 mg/kg DPPE alone were similar. In 50 mg/kg DPPE alone-treated mice, the tumor growth was significantly inhibited in the early stage of tumor growth, compared to that in CDDP alone-treated mice, in addition to a delay of tumor-take. However, even if CDDP was combined with 50 mg/kg DPPE, further inhibition of the tumor growth was not observed. Interestingly, a combination of 25 mg/kg DPPE and CDDP resulted in a marked inhibition of the tumor growth during the treatment period, compared to not only medium alone, but also CDDP alone treatment (Fig. 3A). The survival time of nude mice treated with 25 mg/kg or 50 mg/kg DPPE and 2 mg/kg CDDP was

Table I. Inhibition of KF Cell Proliferation

Drug	IC_{50} (μM)
CDDP	0.4[a]
DPPE	1.7
Tamoxifen	4.4
Clomiphene	1.9

a) Average of three separate experiments. Exposure time, 5 days.

Table II. Inhibition of PKC Activity in KF Cells by Antiestrogens

Drug	IC_{50} (μM)
DPPE	3.0[a]
Tamoxifen	5.5
Clomiphene	6.5

a) Average of three separate experiments. The IC_{50} is the drug concentration required for 50% inhibition of the cytosolic PKC activity in KF cells.

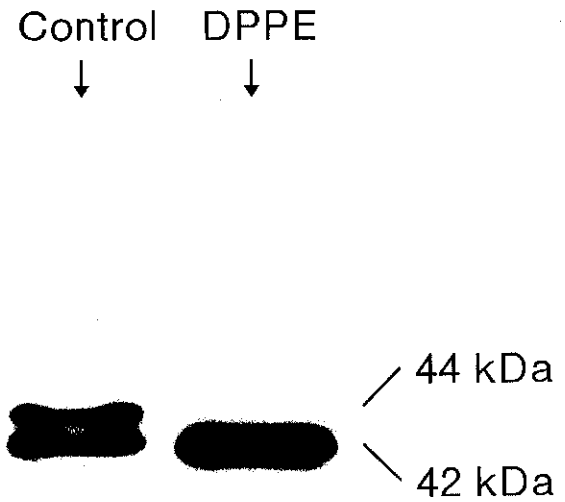

Fig. 2. Inhibition of phosphorylation of MAPK by DPPE. The electrophoretic mobility shift associated with MAPK activation was examined by western blot analysis using an anti-MAPK antibody. DPPE completely inhibited the mobility shift. Control cells were incubated with medium RPMI 1640 containing 10% FCS only for 10 min.

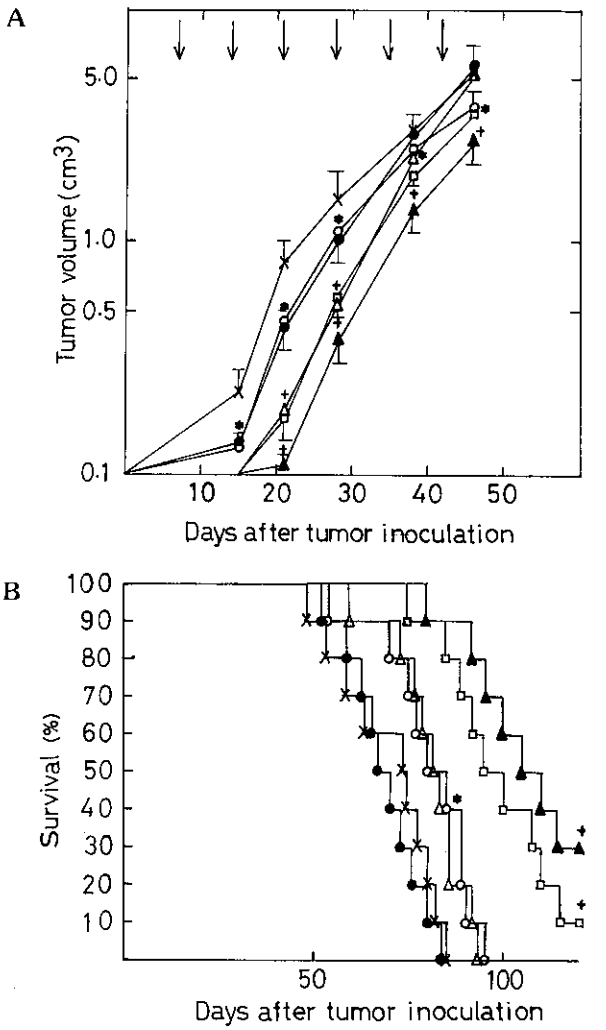

Fig. 3. Combined effects of CDDP and DPPE on the tumor growth and the survival of nude mice bearing human ovarian cancer cells. A, Effects on tumor growth. Treatment was performed from 7 days after tumor inoculation (as shown by arrows). Vertical bars show means±SD from ten mice. $^{*}P<0.05$, compared to medium only. $^{+}P<0.05$, compared to CDDP alone. B, Effects on survival. $^{*}P<0.05$, compared to medium only and 25 mg/kg DPPE only. $^{+}P<0.05$, compared to all single agent groups. ×, medium only; ○, 2 mg/kg CDDP only; ●, 25 mg/kg DPPE only; △, 50 mg/kg DPPE only; ▲, CDDP plus 25 mg/kg DPPE; □, CDDP plus 50 mg/kg DPPE.

significantly ($P<0.05$) longer than that of mice given medium alone, CDDP alone or DPPE alone (Fig. 3B). Three out of 10 mice treated with 25 mg/kg DPPE and CDDP survived with tumor, while one of 10 mice treated with 50 mg/kg DPPE and CDDP survived the experimental course. If treatment was started in nude mice with a smaller tumor load, i.e., on the day after tumor inoculation, the effect of combinations of CDDP and DPPE on the tumor growth and the survival was more marked. Treatment with DPPE (both 25 mg/kg and 50 mg/kg) alone resulted in a delay of tumor-take. Combinations of CDDP and DPPE resulted in further delay of tumor-take, in addition to marked tumor growth retardation (Fig. 4A). Although the survival time of mice treated with DPPE alone was similar to that with CDDP alone, combinations of CDDP and DPPE (25 mg/kg and 50 mg/kg) markedly prolonged the survival time, 3 mice in each group surviving (with tumor) the experimental period (Fig. 4B). These treatments did not cause any adverse side-effects as confirmed by monitoring the hematocrit and the body weight, and by daily inspection.

DISCUSSION

As shown in Fig. 1, the chemical structure of DPPE is similar to those of antiestrogens such as TAM and clomiphene. We have reported antiproliferative effects of clomiphene and TAM alone and in combination with CDDP by using human ovarian cancer cell lines with different sensitivity to CDDP.[2] In addition, we recently demonstrated that relatively low doses (5–10 mg/kg) of DPPE augmented the antitumor activity of CDDP in human ovarian cancer cells with intrinsic or acquired resistance to CDDP.[9] In the present study, we examined

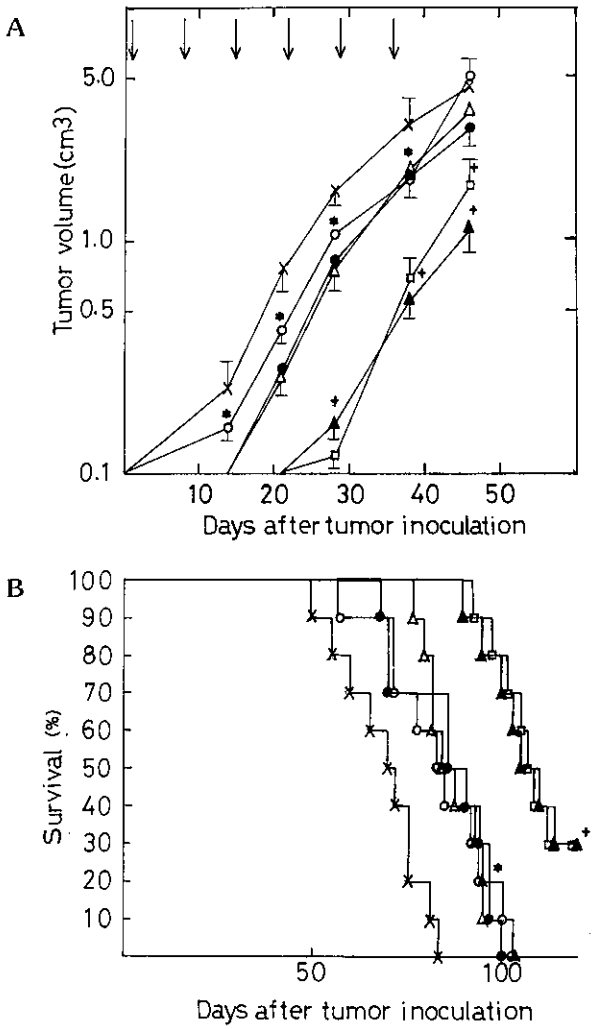

Fig. 4. Combined effects of CDDP and DPPE on the tumor growth and the survival of nude mice bearing human ovarian cancer cells. A, Effects on tumor growth. Treatment was performed from the day after tumor inoculation (as shown by arrows). Vertical bars show means±SD from ten mice. $^{*}P<0.05$, compared to medium only. $^{+}P<0.05$, compared to all treated groups. B, Effects on survival. $^{*}P<0.05$, compared to medium only. $^{+}P<0.05$, compared to all single agent groups.

the effects of DPPE alone on the tumor growth and survival of nude mice bearing human ovarian cancer cells, using relatively higher doses of DPPE.

Regarding the inhibition of human ovarian cancer cell proliferation *in vitro*, DPPE had the same inhibitory effect as clomiphene and TAM (Table I). Since antiestrogens such as TAM and clomiphene have been reported to inhibit cellular PKC activities,[2, 13] we examined the effects of DPPE on the cytosolic PKC activity of KF cells (Table II). DPPE inhibited the PKC activity similarly to TAM and clomiphene. However, DPPE had little effect on the CDDP uptake, unlike TAM or clomiphene. In addition, DPPE completely inhibited the phosphorylation of MAPK induced by serum (Fig. 2). On the other hand, Brandes *et al.*[14] found no correlation between the antiproliferative action of DPPE and PKC-mediated phosphorylation. The method of PKC assay (platelet p47 phosphorylation) and cells (platelets) used in their study are different from the method (a modification of a mixed micelle assay) and cells (human ovarian cancer cells) used in this study. Although the Ras status in the cell line used in this study was not examined, DPPE seems to inhibit the phosphorylation of MAPK in dependently of PKC. The combined effects of DPPE and CDDP can be partly explained by the inhibitory effect of DPPE on the activities of PKC and/or MAPK, while effects of DPPE on the cell-cycle phase, as we have reported previously,[2, 9] may also be involved. These results suggest that DPPE inhibits cell growth through modulation of cellular signal transduction systems.

In an *in vitro* study, we demonstrated previously that DPPE has an additive and somewhat synergistic effect on the antitumor activity of CDDP in CDDP-resistant human ovarian cancer cells.[9] Therefore, we attempted to determine whether such combined effects of DPPE and CDDP can be observed *in vivo* in a tumor-bearing nude mouse model. When treatment was initiated from 7 days after tumor inoculation, 25 mg/kg DPPE was comparable to 2 mg/kg CDDP with regard to inhibition of the tumor growth, while 50 mg/kg DPPE had significantly greater antitumor activity than 2 mg/kg CDDP. With regard to combined effects, combination of 25 mg/kg DPPE with CDDP was more effective than that of 50 mg/kg DPPE (Fig. 3A). When DPPE only was administered once a week, the optimal dose of DPPE seemed to be 25 mg/kg. Although the optimal dose of CDDP seemed to be 3 mg/kg/week (data not shown), a suboptimal dose (2 mg/kg/week) of CDDP was used to confirm the enhancing effect of DPPE on the antitumor activity of CDDP. As regards survival, 25 mg/kg DPPE had no effect, while 50 mg/kg DPPE had an effect comparable to that of 2 mg/kg CDDP. When DPPE was combined with CDDP, the survival was significantly prolonged, compared to not only the medium alone, but also DPPE alone or CDDP alone (Fig. 3B). If treatments were initiated before tumor formation, further delay of the tumor-take was caused even by treatment with DPPE alone. When DPPE was combined with CDDP, marked retardation of the tumor growth was observed (Fig. 4A). The survival time in the case of DPPE alone was equivalent to that in the case of CDDP alone. Further prolongation of the survival was obtained by combining DPPE and CDDP (Fig. 4B). It has been

reported that, in tumor-bearing mice, DPPE (4 to 50 mg/kg) significantly potentiates the cytotoxicity of antineoplastic drugs towards cancer cells and combined DPPE/chemotherapy treatment results in a high *in vivo* cure rate of C-3 fibrosarcoma and B16f 10 melanoma, tumors that are relatively insensitive to DPPE or to chemotherapy alone.[10] In the present study, we obtained similar results using nude mice bearing human ovarian cancer cells.

Despite objective response rates of 60–80% to combination platinum-based chemotherapy, the majority of women with advanced ovarian cancer ultimately develop progressive disease and die of complications of this malignancy.[15] Thus, in addition to the critical need to identify new drugs which have activity in ovarian cancer, it is important to develop rational therapeutic strategies in the relapse/refractory setting which focus on optimizing quality of life and prolonging the time to development of symptomatic disease, as well as extending overall survival of patients with this malignancy. DPPE, a unique drug with both antihistaminic and antiestrogenic actions, may be useful to treat CDDP-resistant ovarian cancer in such clinical settings.

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