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Original Paper

Enhancement of Antitumour Activity of Cisplatin by *N,N*-Diethyl-2-[4-(Phenylmethyl)Phenoxy] Ethanamine·HCl in Human Ovarian Cancer Cells with Intrinsic or Acquired Resistance to Cisplatin

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This study was designed to the elucidate sensitising effects of the intracellular histamine antagonist, *N,N*-diethyl-2-[4-(phenylmethyl)phenoxy] ethanamine·HCl (DPPE) on the antitumour activity of *cis*-diamminedichloroplatinum (II) (CDDP) using human ovarian cancer cell lines with different sensitivities to CDDP (KF, sensitive) KFra (acquired CDDP resistant derived from KF), and KK and MH, intrinsically CDDP resistant. The KF cells were most sensitive to CDDP among cell lines used in this study and followed by MH, KK and KFra showing approximately 3.5, 4.0 and 9.1-fold IC_{50} values to KF, respectively. The acquired CDDP resistant KFra cells were approximately 6.1-fold more sensitive to DPPE than the parent KF cells, while MH and KK cells were more than 10-fold more resistant to DPPE than the KF cells. With regard to the inhibition of human ovarian cancer cell proliferation, phenyltoloxamine and L-histidinol were 5–2500-fold less cytotoxic than DPPE. Analysis of flow cytometry (FCM) revealed that with concentrations based on the IC_{50} to KF and KFra cells, DPPE resulted in G_2-M accumulation in the KF (but not KFra) cells in a time-dependent manner during the course of 48 h incubation time. In addition, from a median effect analysis, DPPE seemed to have additive and somewhat synergistic effects on the antitumour activity of CDDP in KK and MH cells with intrinsic CDDP resistance, while minor antagonism in KFra cells with acquired CDDP resistance was observed. Although DPPE alone did not significantly inhibit the tumour growth of nude mice bearing KF cells, combinations of DPPE with CDDP resulted in improved survival compared with treatment with only CDDP. Adverse side-effects, as confirmed by monitoring haematocrit and the body weight were not observed during the experimental period. These results suggest that DPPE may be of clinical use for the treatment of intrinsically refractory ovarian carcinoma when combined with CDDP.

Key words: *N,N*-diethyl-2-[4-(phenylmethyl)phenoxy] ethanamine · HCl, *cis*-diamminedichloroplatinum (II), human ovarian cancer cells, cisplatin resistance

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INTRODUCTION

RESISTANCE TO chemotherapy limits the efficacy of treatment for ovarian cancer. Although platinum-based combination chemotherapy is associated with a 60–80% clinical objective response rate [1] in patients with advanced-stage disease, the 5 year survival for this population is approximately 10–20%. Virtually all of these patients die with chemotherapy-refractory cancer. In order to improve the survival of such patients, enhancement of the antitumour activity of CDDP is of great importance.

Since the paradiphenylmethane tamoxifen (TAM) derivative, *N,N*-diethyl-2-[4-(phenylmethyl)phenoxy] ethanamine HCl (DPPE), was synthesised by Brandes and Hermonat

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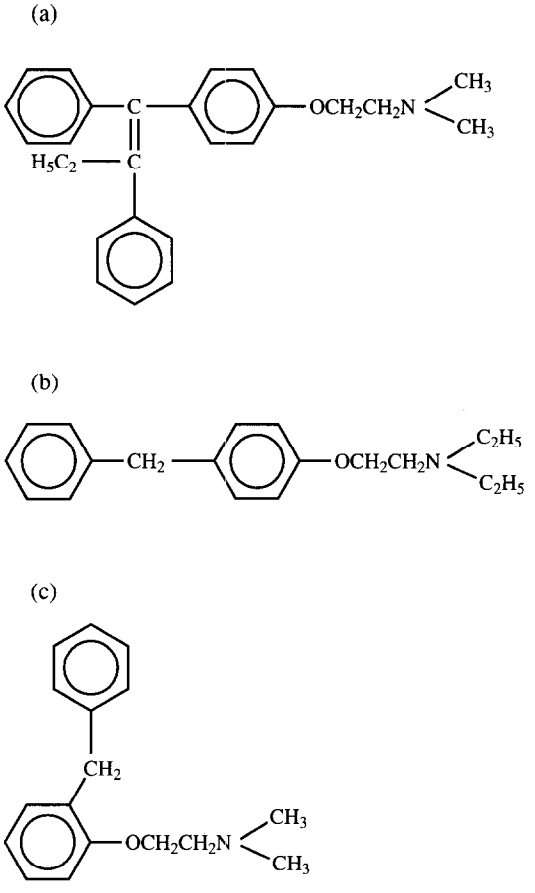

Figure 1. Chemical structures of tamoxifen and antihistamines. (a) Tamoxifen, (b) *N,N*-diethyl-2-[4-(phenylmethyl)-phenoxy]ethanamine · HCl (DPPE), (c) phenyltoloxamine.

[2], (see Figure 1 for structure), it has been suggested that intracellular histamine mediates cell proliferation. A diphenylmethane derivative anti-oestrogen binding site (AEBS) ligand, DPPE, like TAM, is antiproliferative *in vitro* [3] and binds with high affinity to AEBS (K_i $\sim 50 \times 10^{-9}$M) [2]. Unlike TAM, DPPE does not interact with calmodulin [4] or bind to the oestrogen receptor [2]. Several studies have demonstrated that potentiation of CDDP cytotoxicity by calmodulin inhibitors and antioestrogens occurs through enhanced drug uptake [5–7] or through a mechanism involving inhibition of the incision step of DNA repair [8]. Observations that some AEBS ligands, including TAM and phenothiazines inhibit binding of tumour-promoting phorbol esters and/or protein kinase C action [9, 10] suggest a mechanism for the antiproliferative effect of DPPE. We have also reported that antioestrogens (clomiphene and TAM) and phorbol esters enhance the antitumour effects of *cis*-diamminedichloroplatinum (II) (CDDP) through inhibition of protein kinase C [6, 11]. Thus, we attempted to determine the effects of DPPE and CDDP *in vitro* and *in vivo* using human ovarian cancer cell lines with different sensitivities to CDDP.

MATERIALS AND METHODS

Chemicals

CDDP was obtained from Bristol–Myers Squibb Co. Ltd. (Tokyo, Japan). Phenyltoloxamine and L-histidinol were purchased from Sigma (St. Louis, MO). DPPE was kindly supplied by Dr Lorne J. Brandes. These drugs were dissolved and diluted with medium or saline to the desired concentrations prior to use.

Cell lines

KF cells were established in 1982 from the tissue of a patient with a serous cystadenocarcinoma of the ovary [12]. CDDP-resistant KFra cells were obtained by repeated exposure of the KF cells to escalating doses of CDDP [13]. The KK and MH cells were established from ascites of a patient with a clear cell carcinoma of the ovary and with a serous cystadenocarcinoma of the ovary, respectively, who did not respond to CDDP-based combination chemotherapy [6, 11]. All cell lines were incubated in RPMI 1640 medium supplemented with 10% fetal calf serum, 2mM glutamine, 100 units penicillin/ml and 100 μg streptomycin/ml (GIBCO, Grand Island, New York, U.S.A.) in 5% CO_2 at 37°C. The medium was changed every 3 days, and the cells were passaged when they reached confluence. Characteristics of cell lines used in this study are described in Table 1

Drug sensitivity

To determine concentrations of CDDP and antihistamines required for 50% inhibition of KF, KFra, MH and KK cell proliferations *in vitro* (IC_{50}), we seeded 5×10^2 cells for KF and KFra and 2×10^3 cells for MH and KK in 96-well flat-bottomed microtest-plates (Becton–Dickinson Co., Mountain View, California, U.S.A.), and incubated in the absence or presence of CDDP and antihistamines in a humidified atmosphere of 5% CO_2 at 37°C. After incubation for 5 days, the cytotoxicity of CDDP and antihistamines on each cell was determined using a crystal violet staining method [14]. Briefly, an equal volume of 10% for-

Table 1. Characteristics of ovarian cancer cell lines used in this study

Cell line	Sensitivity to CDDP	Transplant to nude mice	Doubling time (h)	Plating efficiency (%)	Protein (μg/10^6 cells)	CDDP uptake (ng/mg protein)
KF	Sensitive	100%	15 ± 2*	59.2 ± 4.2	126 ± 19	86.6 ± 18.7
KFra	Acquired r†	40–70%	19 ± 1	61.5 ± 0.2	174 ± 50	53.6 ± 8.6
MH	Intrinsic r‡	0%	36 ± 3	46.0 ± 5.2	250 ± 55	47.3 ± 7.5
KK	Intrinsic r	0%	29 ± 2	30.2 ± 6.9	308 ± 59	60.0 ± 13.4

* Mean ± SD from quadruplicate experiments.

† Acquired resistance derived from KF cells.

‡ Intrinsic resistant cells established from primary tumour of patients who did not respond to CDDP-based combination chemotherapy. CDDP uptake was measured by atomic absorption spectroscopy.

malin phosphate buffered saline containing 0.2% crystal violet was added into each well and left at room temperature for 20 min. After washing twice with distilled water and drying at room temperature, absorbance of stained cells in each well was measured at 590 nm by an automatic microtest-plate reader (Multiscan MCC/340, Titertek, Flow Laboratories Inc., Virginia, U.S.A.). The 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 CDDP and antihistamines that inhibited the growth of cells to the level of 50% of the control growth (IC_{50}) was determined from dose–response curves.

Median effects analysis

The cytotoxicity data of DPPE, CDDP and combinations of these drugs in constant molar ratios were analysed by median effect analysis [15]. Briefly, the analysis compares the effects of drug combinations to the effects of individual drugs across the entire dose–effect range. Cytotoxicity data were then fitted to regression lines, and the concentration of each drug which produced a given level of cytotoxicity (fractional effect, Fa) alone or in combination was determined. The combination index (CI) for a given Fa was calculated as: $CI = d^1/D_1 + d^2/D_2$, where D_1 and D_2 are the doses of drugs 1 and 2, which by themselves produce a given Fa (i.e. IC_{50}); d_1 and d_2 are the doses which produce the same Fa in combination. CI = 1 indicates zero interaction (additive cytotoxicity), CI < 1 indicates synergy and CI > 1 indicates antagonism.

Flow cytometry

Cell cycle phase distribution analysis was performed with a FCS-1 multiparametric cell sorter (Japan Spectroscopic Co., Ltd., Tokyo, Japan) using propidium iodide-stained nuclei of the cells. Briefly, control and treated cells were washed twice with ice-cold 0.85% sodium chloride solution and the cell pellet was resuspended in hypotonic propidium iodide staining solution [16]. The nuclei were maintained at 4°C for 24 h in the staining solution prior to analysis for cell cycle traverse perturbation. At least 10^4 nuclei were analysed in each sample, and the fractions of cells G_1-G_0, S, and G_2-M of the cell cycle were determined as reported previously [17].

Nude mice

Approximately 6-week-old female BALB/c nude mice were obtained from Japan Clea Laboratories, Tokyo, Japan, and maintained in a pathogen-free environment. The animals were inspected daily and tumour growth was determined with a caliper. When necessary, the animals were killed and dissected. The tumour tissues were fixed in formalin for histological examination. The larger tumours (more than 2 cm in diameter) contained a larger necrotic area in the centre. Distant metastases were not observed during the experimental period. Unless treated, all mice with tumours died of tumour burden within 100 days of tumour inoculation.

In vivo treatment

As shown in Table 1, KK and MH cells were not heterotransplantable to nude mice. The transplantability of KFra cells to nude mice was unstable showing 40–70%. Therefore, KF cells with 100% transplantability were selected for the following *in vivo* experiments. To determine the combined effect of CDDP and DPPE on tumour growth and survival time, 5×10^5 KF cells were inoculated s.c. into the right flank of nude mice. From 14 days after tumour inoculation, treatment with CDDP and DPPE was initiated 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 ($n = 10$), 5 mg/kg DPPE alone was administered i.p. once a week for 6 weeks; group 4 ($n = 10$), 10 mg/kg DPPE alone was administered i.p. once a week for 6 weeks; group 5 ($n = 10$), 2 mg/kg CDDP and 5 mg/kg DPPE were simultaneously administered i.p. once a week for 6 weeks; group 6 ($n = 10$), 2 mg/kg CDDP and 10 mg/kg DPPE were simultaneously administered i.p. once a week for 6 weeks. Each injection was given in a 0.15 ml volume. In preliminary *in vivo* experiments using KF cells, we examined the tolerable optimal dose of CDDP. When CDDP was administered i.p. once a week, 3 mg/kg of CDDP seemed to be optimal with regard to the effect on both tumour growth and survival. Treatment with more than 3 mg/kg of CDDP although, markedly inhibited tumour growth and caused notable toxicity, survival time was not significantly prolonged. Since the aim of this study was to potentiate the antitumour activity of CDDP by DPPE, a suboptimal dose of CDDP (2 mg/kg) was selected. Tumour growth was determined by the measurement of diameters of the tumour nodule in two dimensions with a caliper once a week. The tumour 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 haematocrit tubes every week and the haematocrit values and body weight were recorded for monitoring the side-effects of drugs. Since these experiments were repeated and similar results were obtained, results from the first experiment are presented.

Statistical analysis

Where indicated, statistical significance was tested using a two-tailed Student's *t*-test; a p value of <0.05 was considered significant.

RESULTS

Effects of CDDP and antihistamines on the proliferation of human ovarian cancer cell lines

As shown in Table 2 , the KF cells were most sensitive to CDDP among four cell lines used in this study. The IC_{50} ratio to the KF cells was 3.5 for MH cells, 4.0 for KK cells and 9.1 for KFra cells, respectively. The IC_{50} of DPPE for CDDP-resistant KFra cells was 0.27 μM, being around 6-fold more sensitive to DPPE than the parent KF cells, while MH and KK cells were more than 10-fold, more resistant to DPPE than KF cells. With regard to the inhibition of human ovarian cancer cell proliferation, phenyltoloxamine and L-histidinol were 5–2500 bold less antiproliferative than DPPE. To determine the mechanism of the antiproliferative effect of DPPE, we performed flow cytometry (FCM) analysis using the CDDP-resistant KFra and the

Table 2. IC_{50} of CDDP and antihistamines to human ovarian cancer cell lines

Cell line	CDDP	DPPE	Phenyltoloxamine	L-histidinol
KF	0.37*	1.66	58.9	555.5
KFra	3.35	0.27	61.6	666.4
MH	1.28	19.72	97.1	827.1
KK	1.47	18.57	97.3	713.8

* IC_{50} (μM), average from three separate experiments. Exposure time, 5 days.

parent KF cells (Figure 2). In the KF cell line, concentrations of DPPE around the IC_{50} (1.66 μM) resulted in G_2-M accumulation in a time-dependent rather than a dose-dependent manner. Similarly, in the DPPE sensitive KFra cells, the G_2-M arrest seemed to occur at more than 2-fold concentrations of the IC_{50} (0.54 μM). In addition, to

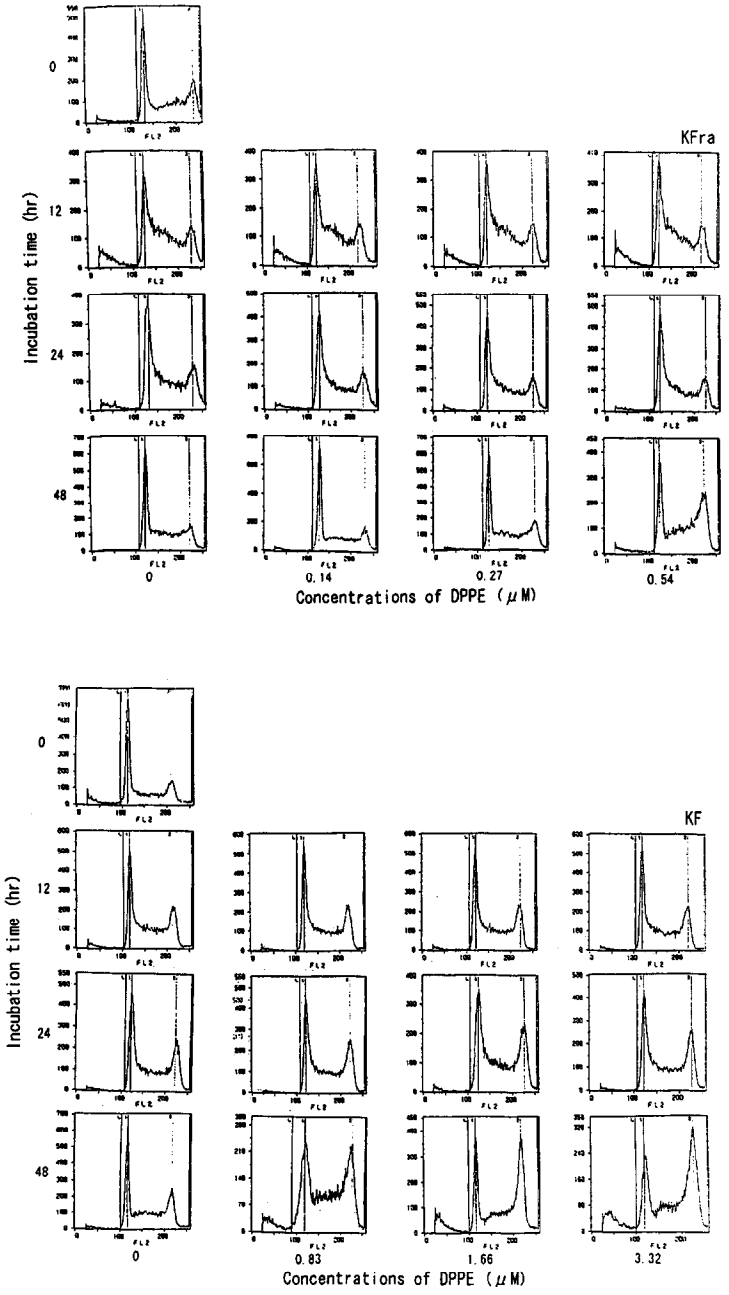

Figure 2. Effect of DPPE on the cell cycle phase of KF and KFra cell lines. After these preconfluent cells were incubated for 12, 24 and 48 h with concentrations based on the IC_{50} of DPPE to KF and KFra cells, the cells were harvested and washed three times with fresh medium. Preincubated (0 h) cells and incubated cells were analysed by flow cytometry.

Table 3. Median effect analysis of the interaction between DPPE and CDDP in human ovarian cancer cell lines

Cell lines	Combination index (CI)		
	Fa* = IC_{80}	Fa = IC_{50}	Fa = IC_{20}
MH	0.92 ± 0.03†	0.91 ± 0.02	0.95 ± 0.01
KK	0.78 ± 0.01‡	1.00 ± 0.01	1.06 ± 0.02
KF	1.00 ± 0.03	1.01 ± 0.02	1.03 ± 0.01
KFra	1.21 ± 0.02	1.33 ± 0.04	1.75 ± 0.27

* Fractional effect. † Mean ± SD from three separate experiments. ‡ $P < 0.01$ (Student's t-test), compared to CI = 1.

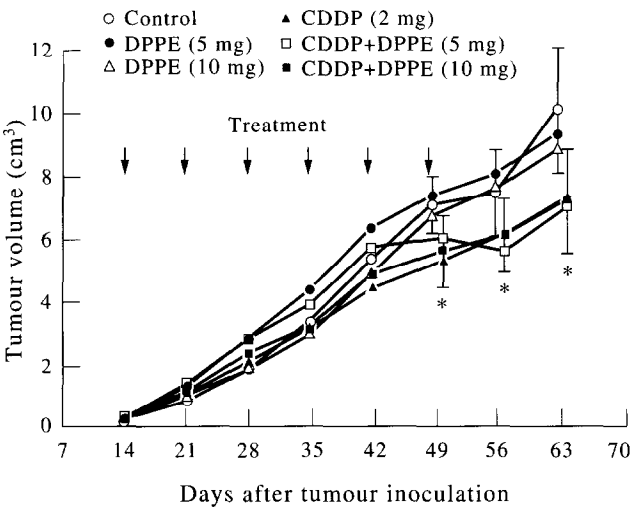

Figure 3. Effects of DPPE and CDDP on the tumour growth of KF cells inoculated into nude mice. Bars show the mean ± SD from 10 mice. *$P < 0.01$ (Mann–Whitney U-test), compared to control (medium alone treated) mice.

examine the combined effect of CDDP and DPPE, we carried out a median effect analysis. In KFra cells with acquired resistance to CDDP, DPPE seemed to have a somewhat antagonistic effect on antitumour activity of CDDP, while as additive effect was observed in the parent KF cells (Table 3). In KK and MH cells with intrinsic resistance to CDDP, DPPE seemed to have essentially additive effects on antitumour activity of CDDP (Table 3).

Adjuvant effects of DPPE on CDDP in KF cell-bearing nude mice

Although DPPE alone did not have any effect on tumour growth *in vivo*, CDDP alone inhibited tumour growth after 49 days of tumour inoculation. When DPPE was combined with CDDP, a significant greater effect on tumour growth was observed (Figure 3) and survival time was significantly prolonged compared to that of not only untreated controls but also those given only CDDP (Table 4). As confirmed by monitoring the hematocrit and body weight, the doses of CDDP and DPPE used in this study did not result in any adverse effects (data not shown), and no gross pathological effects were noted.

DISCUSSION

In the present study, we have demonstrated that DPPE, of three antihistamines used, had the most marked antiproliferative effect on the human ovarian cancer cell lines (Table 2). The histamine H_1-receptor antagonists, phenyltoloxamine and L-histidinol, were 5–2500-fold less cytotoxic to the human ovarian cancer cell lines than DPPE. It is noteworthy that DPPE had activity in KFra cells with acquired resistance to CDDP, while MH and KK cells with intrinsic resistance to CDDP were more than 10-fold less sensitive to DPPE than CDDP. Although the sensitivity of KF and KFra cells to DPPE seemed to be relatively high, that of MH and KK cells was similar to that reported for MCF-7 cells [18]. The IC_{50} values of these cell lines to L-histidinol were also consistent with this previous report [18]. In addition, we attempted to elucidate

Table 4. Adjuvant effects of DPPE or CDDP on the survival of nude mice bearing human ovarian cancer cells

Treatment (N)	Survival time (days)	% Increase of life span
Untreated (10)	73 ± 5*	
DPPE 5mg/kg (10)	90 ± 8	23.2†
DPPE 10mg/kg (10)	68 ± 6	−6.8
CDDP 2mg/kg (10)	105 ± 7	43.8 16.7‡ 54.4§
CDDP + DPPE 5mg/kg (10)	123 ± 5‖	68.5 36.7‡ 80.9§ 17.1¶
CDDP + DPPE 10mg/kg (10)	135 ± 4‖	84.9 50.0‡ 98.5§ 28.6¶

* Mean ± SD. † Compared to untreated group. ‡ Compared to DPPE 5mg/kg only. § Compared to DPPE 10mg/kg only. ‖ $P < 0.01$, compared to CDDP only. ¶ Compared to CDDP only.

the effect of DPPE on the cell cycle phase using KFra cells with acquired resistance to CDDP and the parent KF cells. As shown in Figure 2, the cell cycle phase of the parent KF cells was arrested in the G_2-M phase in a time-dependent but not a dose-dependent manner around the IC_{50} concentrations of DPPE. However, the cell cycle phase of the KFra cells was not affected by the IC_{50} concentrations of DPPE. Since the doubling time of KFra cells was longer than that of KF cells, a longer incubation of KFra cells with DPPE may be necessary to obtain cell-cycle arrest. To determine the combined effects of CDDP and DPPE on the human ovarian cancer cell proliferations, the median effect analysis was performed (Table 3). DPPE had a somewhat antagonistic action with CDDP in acquired CDDP-resistant KFra cells with high sensitivity to DPPE, while it had additive and/or somewhat synergistic actions with CDDP in the KK and MH cells with intrinsic resistance to CDDP. Thus, some caution must be paid when combining DPPE with CDDP in the treatment of certain tumours, especially with acquired resistance to CDDP. However, we can expect a synergistic effect into a combination of DPPE and CDDP on some tumours with intrinsic resistance to CDDP.

In addition, we investigated the effect of DPPE alone on tumour growth *in vivo* and examined whether DPPE could augment CDDP antitumour activity. DPPE (5 or 10 mg/kg) alone did not significantly inhibit the tumour growth of nude mice bearing KF cells. Although only 2 mg/kg of CDDP significantly inhibited tumour growth after 49 days of tumour inoculation, the tumour growth curve was not affected if CDDP was combined with DPPE (Figure 3). The lack of antitumour responsiveness with CDDP and DPPE may be due to inadequate tumour levels of DPPE being reached (possibly bound to proteins?). Accordingly, further animal studies are ongoing using higher doses of DPPE. However, when CDDP was combined with 10 mg/kg DPPE, a significant prolongation of survival occurred compared with both untreated controls, DPPE only or CDDP only treated mice, suggesting a significantly greater effect of CDDP or CPPE (Table 4). Edelstein [19] has demonstrated that both L1210 leukaemia and colon 26 adenocarcinoma cells are more efficiently killed by combinations of L-histidinol and cisplatin, L-histidinol preventing the toxicity of cisplatin. This may explain why, despite the failure of DPPE to augment the inhibitory effects of CDDP on tumour growth, DPPE could significantly prolong the survival of nude mice treated with CDDP alone. Similarly, DPPE has also been shown to increase the therapeutic index of antineoplastic drugs in mice [18]. It has been reported that potentiation of CDDP cytotoxicity by calmodulin inhibitors results from enhanced drug uptake or through a mechanism involving inhibition of the incision step of DNA repair [5, 8]. As shown in Table 1, the mechanism of CDDP resistance in cell lines used in this study seemed to be partly due to reduced CDDP accumulation levels. We have previously reported that anti-oestrogens (tamoxifen and clomiphene) can potentiate antitumour activity of CDDP in the CDDP-resistant cell lines by enhancing CDDP-uptake [6]. Since DPPE has been known to have anti-oestrogenic action, DPPE may reverse CDDP resistance by enhancing CDDP accumulation. Recently, Brandes and associates [20, 21] have also suggested that DPPE may increase the therapeutic index of certain chemotherapy drugs, although the results are preliminary and need confirmation.

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