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Sensitisation of Human Ovarian Carcinoma Cells to *Cis*-diamminedichloroplatinum (II) by Amphotericin B *In vitro* and *In vivo*

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Human ovarian carcinoma cells (HRA) were sensitised to *cis*-diamminedichloroplatinum (II) (CDDP) 2.7-, 5.5- and 12.1-fold by treatment with amphotericin B (AMB) at concentrations of 2.1, 5.4 and 10.8 μM, respectively. Moreover, intracellular accumulation of platinum after a 2-h exposure to CDDP was increased significantly with AMB treatment. We prepared HRA cell-inoculated nude mice as an experimental therapeutic model for human advanced ovarian carcinoma. Ascites was evident after 7 to 9 days of intra-peritoneal (i.p.) inoculation of HRA cells, and mice died due to intra-abdominal carcinomatosis after 11 to 14 days [mean survival time (MST): 12.4 ± 1.1 days]. Treatment with AMB (2.0 mg/kg) alone 4 days after inoculation increased MST by only 1.4 days. Simultaneous treatment with CDDP (1.0 to 2.0 mg/kg) and AMB (0.5 to 2.0 mg/kg) produced a significant increase in MST compared to treatment with CDDP alone. Maximal MST (30.1 days) was observed by treatment with 2.0 mg/kg CDDP plus 2.0 mg/kg AMB, whereas MST with 2.0 mg/kg CDDP alone was 16.4 days. A further drug accumulation study demonstrated that platinum accumulation in tumour tissues in nude mice treated with CDDP and AMB increased significantly compared to treatment with CDDP alone. These results indicate that intraperitoneal combination chemotherapy with CDDP and AMB is effective in an experimental animal model of advanced ovarian carcinoma.

Key words: *cis*-diamminedichloroplatinum (II), amphotericin B, ovarian carcinoma, biochemical modulation, chemotherapy

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INTRODUCTION

CIS-DIAMMINEDICHLOROPLATINUM (II) (CDDP) is an effective anticancer drug, and is in wide use clinically, especially for testicular, ovarian, head and neck, bladder and lung cancers [1]. CDDP has increased the 5-year survival rate in ovarian carcinoma, and enhancing CDDP cytotoxicity may increase the survival rate further. Although several approaches have been followed to enhance cytotoxicity of anticancer drugs, including CDDP, these attempts have met with limited success because the modifying agents are too toxic to normal as well as tumour tissues. The polyene antibiotic amphotericin B (AMB) has a unique mechanism of action related to its affinity for sterols in the cell membrane [2]. Studies have shown that AMB binds to cholesterol and causes 0.4–1.0 nm pores in the cell membrane, increasing permeability and facilitating uptake of various molecules [3–5]. Medoff and colleagues indicated that AMB could enhance the effect of cytotoxic agents in certain tissue culture systems in mice with AKR leukaemia [6–8]. In addition, several studies suggested that treatment with AMB significantly enhances cytotoxicity of drugs, such as nitrosourea, actinomycin D, vincristine and doxorubicin [9–13]. These observations in animal models and in cell culture systems have led to combinations of AMB and cytotoxic agents in phase I and phase II trials [14–16]. However, the combination of AMB and CDDP has not been reported *in vivo*, although CDDP is the most effective anticancer drug in ovarian carcinoma.

In this study, we examined the effectiveness of AMB on CDDP cytotoxicity and the accumulation of CDDP *in vivo* and *in vitro*.

MATERIALS AND METHODS

Chemicals

AMB, in the form of fungizone, was obtained from Bristol-Myers Squibb and was dissolved in a 5% dextrose solution immediately before use. CDDP was obtained from Nippon Kayaku (Tokyo, Japan). Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, U.S.A.). This reagent system combines the well known reaction of protein with Cu^{2+} in an alkaline medium with a highly sensitive and selective detection reagent for Cu^{1+}, namely BCA.

Nude mice

Seven-week-old female BALB/c nude mice, weighing 18–20 g, were purchased from Charles River (Hino, Tokyo),

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and maintained in a pathogen-free environment. Mice were allowed food and water *ad libitum*.

Cells

HRA cells, derived from the ascites of a patient with serous cystadenocarcinoma of the ovary, were kindly provided by Dr Y. Kikuchi (National Defense Medical College, Saitama, Japan). The cells were incubated in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, 0.17 mM penicillin G and 69 nM streptomycin in 5% CO_2 at 37°C.

Drug sensitivity studies

Drug sensitivity of the cells was examined by 3-[(4,5)]-dimethylthiazol-2-yl-2,5 diphenyl tetrazolium bromide (MTT, Sigma, St Louis, Missouri, U.S.A.) assay as described previously [17]. Two thousand cells were seeded into a 96-well microplate (Falcon 3072, Becton Dickinson, New Jersey, U.S.A.), and incubated continuously with various concentrations of drugs in a humidified atmosphere of 5% CO_2 at 37°C. After the addition of 50 μl tetrazolium salt (MTT), the culture was continued for 4 h at 37°C. The supernatant was then aspirated, and the resulting pigment was dissolved in dimethyl sulfoxide. Absorbance at 550 nm was measured using Easy Reader (EAR 400 RW, SLT-Labinstruments, Austria). Twelve wells were used for each drug concentration, and the mean was determined by repeating the measurement in each culture at least three times.

An isobologram was drawn by two curves of mode 1 and mode 2, with the points plotted in the coordinate generally corresponding to concentrations of two drugs that show certain survival fractions [18]. In this study, the survival fraction was fixed at 50% and the drug concentrations were shown by IC_{50} units. Points on mode 1 coincide with the two drug concentrations that inhibit 50% based on a supposition that the drugs work independently, while points on mode 2 correspond to the two-drug concentrations that inhibit 50% on a supposition that the drugs have identical action.

Survival studies in nude mice

For determining the effect of CDDP *in vivo*, 1.0, 1.5 or 2 mg/kg CDDP was injected intraperitoneally (i.p.) 3, 5, 7 and 9 days after an i.p. transplantation of 10^7 HRA cells in nude mice. To examine the effect of AMB alone, 2 mg/kg AMB was injected i.p. following the same procedure. Furthermore, for determining the effect of AMB on CDDP, both drugs were injected simultaneously in the same procedure (CDDP 1.5 mg/kg plus AMB 0.5, 1.0 and 2.0 mg/kg; CDDP 1.0 mg/kg plus AMB 2.0 mg/kg; CDDP 2 mg/kg plus AMB 2.0 mg/kg). Each experimental group contained six to 10 mice.

Side-effects of AMB and CDDP

Haematological and biological examinations were performed 5 and 10 days after administration of CDDP (2.0 mg/kg) with or without AMB (2.0 mg/kg), four times every other day. Each experimental group contained four nude mice.

Drug accumulation studies in vitro

Exponentially growing cells were incubated in fresh RPMI 1640 medium containing 10% fetal bovine serum and CDDP with or without AMB at various concentrations for 2 h. After washing with phosphate buffered saline twice, cells were collected by trypsinisation and were stored at −20°C. Intracellular platinum accumulation was measured by a modified method of Pera and colleagues [19]. Cell pellets were mixed with 16 N HNO_3 and then evaporated until dry. Each sample was dissolved in 0.1 N HNO_3 and determined for platinum by flameless atomic absorption spectrometry (AA-8500 MK II, Nippon Jarrell-Ash Co. Ltd.). The intracellular platinum accumulation was normalised to the cellular protein content estimated by BCA protein assay kit.

Drug accumulation studies in vivo

Drugs were injected i.p. simultaneously at a dose of CDDP 1.5 mg/kg and AMB 1.0 mg/kg, and CDDP 1.5 mg/kg and AMB 2.0 mg/kg, 10 days after the i.p. transplantation of 10^7 HRA cells in nude mice. Mice were sacrificed and tumour tissues were removed 2 h after administration. After washing with phosphate buffered saline twice, tumour tissues were stored at −20°C. Platinum accumulation was measured by the atomic absorption method described above. Each experimental group contained four nude mice.

Statistical analysis

Results are presented as the mean ± S.D. The significance of difference in survival time was determined by the Wilcoxon's test and other statistical analysis was performed by the Student's *t*-test.

RESULTS

Effect of AMB in HRA cells

The cytotoxicity of AMB was investigated in human ovarian carcinoma (HRA) cells (Figure 1). AMB alone had no antitumour effect on cell survival at concentrations below 3.2 μM, while higher concentrations were increasingly cytotoxic. The concentration of CDDP alone required for 50% inhibition of growth (IC_{50}) was 3.3 μM and treatment with AMB sensitised HRA cells to CDDP in a dose-dependent manner (Figure 2a). Dose modification factors (DMF), a ratio of IC_{50} for CDDP to that in addition to AMB, were 2.7, 5.5 and 12.1 at a concentration of 2.1, 5.4 and 10.8 μM of AMB, respectively. An isobologram was drawn according to sensitivity tests of cotreatment with CDDP and AMB (Figure 2b). Since the points plotted in the coordinate were below the mode 1 curve, the treatment with CDDP and AMB was judged to have a synergistic effect.

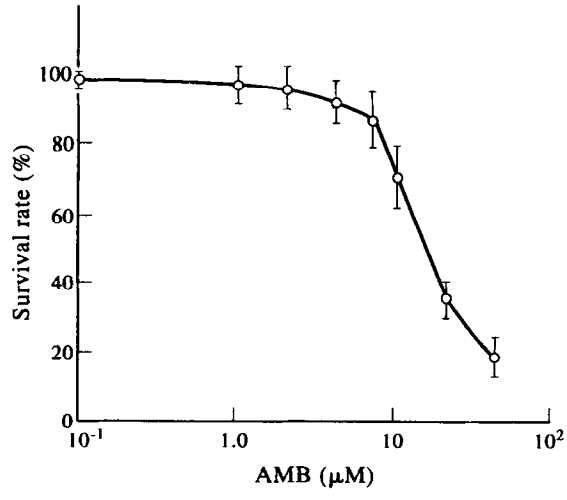

Figure 1. Inhibition of HRA cell growth by AMB as determined by MTT assay. Points are the mean values for six independent experiments. Bars indicate the S.D.

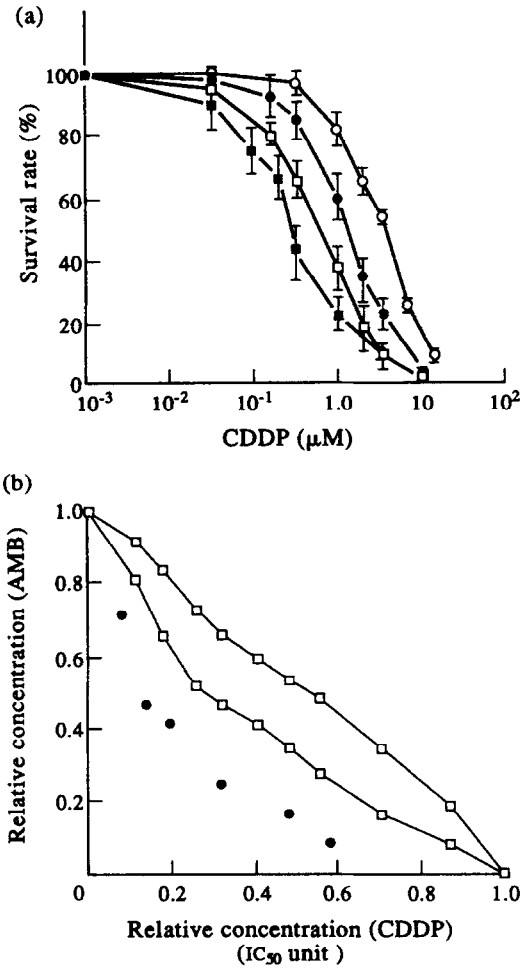

Figure 2. (a) Inhibition of cell growth by CDDP with AMB. HRA cells were incubated with AMB 0 (○), 2.1 (●), 5.4 (□) and 10.8 μM (■). Points are the mean values for six independent experiments. Bars indicate the S.D. (b) Isobologram is drawn by two curves of mode 1 and mode 2, and points plotted in the coordinate generally correspond to concentrations of two drugs that show certain survival fractions. The X and Y axes were expressed as relative concentrations (IC_{50} unit).

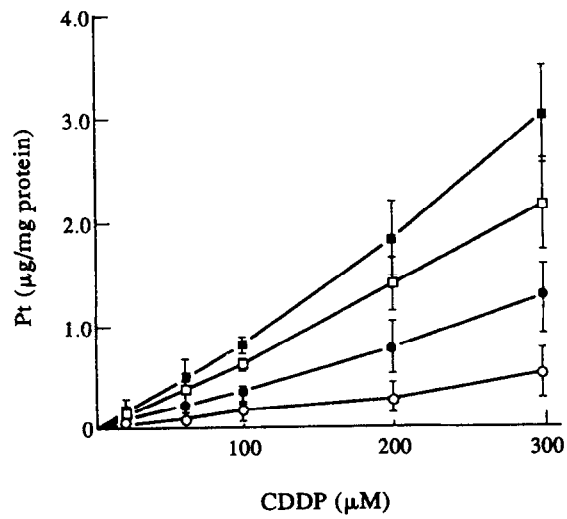

Figure 3. Intracellular accumulation of CDDP after 2 h exposure to various concentrations of CDDP with AMB. HRA cells were incubated with AMB 0 (○), 2.1 (●), 5.4 (□) and 10.8 μM (■). Each point represents the mean of four independent experiments.

CDDP accumulation studies in HRA cells

Intracellular accumulation of CDDP in HRA cells was determined by atomic absorption spectrophotometry. HRA cells were incubated with 20, 60, 100, 200 or 300 μM CDDP with or without 2.1, 5.4 or 10.8 μM of AMB for 2 h. The intracellular platinum accumulation increased in a dose-dependent manner, and was significantly enhanced 2.2, 3.9 and 5.4 times by co-incubation with 2.1, 5.4 and 10.8 μM of AMB, respectively (Figure 3). These data suggested that the enhancing effect of AMB on CDDP cytotoxicity was partially due to increased intracellular platinum accumulation.

Effect of AMB and CDDP on survival in nude mice

Figure 4 shows the effects of CDDP plus AMB on survival of BALB/c nude mice bearing HRA cells. Tumour-bearing mice receiving no drug were used as controls. Ascites was evident from 7 to 9 days after i.p. inoculation of 10^7 tumour cells, and mice died due to intra-abdominal carcinomatosis from 11 to 14 days [mean survival time (MST) 12.4 ± 1.1 days]. Treatment with 2.0 mg/kg of AMB had no antitumour effect (MST 13.8 ± 1.8 days). Treatment with 1.5 mg/kg of CDDP extended the survival time for only 2 days (mean survival time: 14.1 ± 1.3 days), whereas simultaneous treatment with 1.5 mg/kg of CDDP plus AMB produced a dose-dependent, significant increase in survival time (MST 0.5 mg/kg, 16.8 ± 2.3 days; 1.0 mg/kg, 21.3 ± 3.5 days; 2.0 mg/kg, 25.1 ± 4.5 days) compared to CDDP alone.

CDDP alone produced a dose-dependent increase in survival time (Figure 5). However, MST was extended by only 4 days even by 2 mg/kg of CDDP. Simultaneous treatment with 2.0 mg/kg of AMB produced a further significant increase in survival time (MST CDDP 1.0 mg/kg, 19.6 ± 3.4 days; CDDP 1.5 mg/kg, 25.1 ± 4.5 days; CDDP 2.0 mg/kg, 30.1 ± 9.4 days) compared to CDDP alone. AMB was more effective in combination with higher concentrations of CDDP in tumour-bearing mice. There were no significant differences in the results of the haematological and biochemical examinations among the control, CDDP 2.0 mg/kg, and CDDP 2.0 mg/kg with AMB 2.0 mg/kg groups, as shown in Table 1.

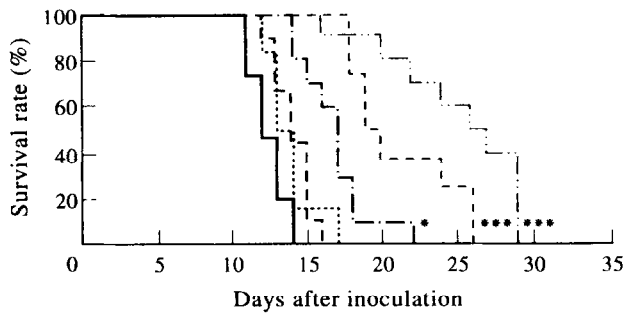

Figure 4. Therapeutic effect of combination treatment with CDDP plus AMB on mean survival time. Tumour-bearing nude mice were untreated (———) or treated with: AMB 2.0 mg/kg alone (----); CDDP 1.5 mg/kg alone (— —); or CDDP 1.5 mg/kg plus AMB 0.5 (———·——), 1.0 (–––) or 2.0 mg/kg (————). Data presented were from a representative experiment performed with more than six mice in each group. The significant difference in survival was determined by Wilcoxon's test. *Significantly different ($P < 0.05$) from the group treated with CDDP alone. **Significantly different ($P < 0.01$) from the group treated with CDDP alone. ***Significantly different ($P < 0.001$) from the group treated with CDDP alone.

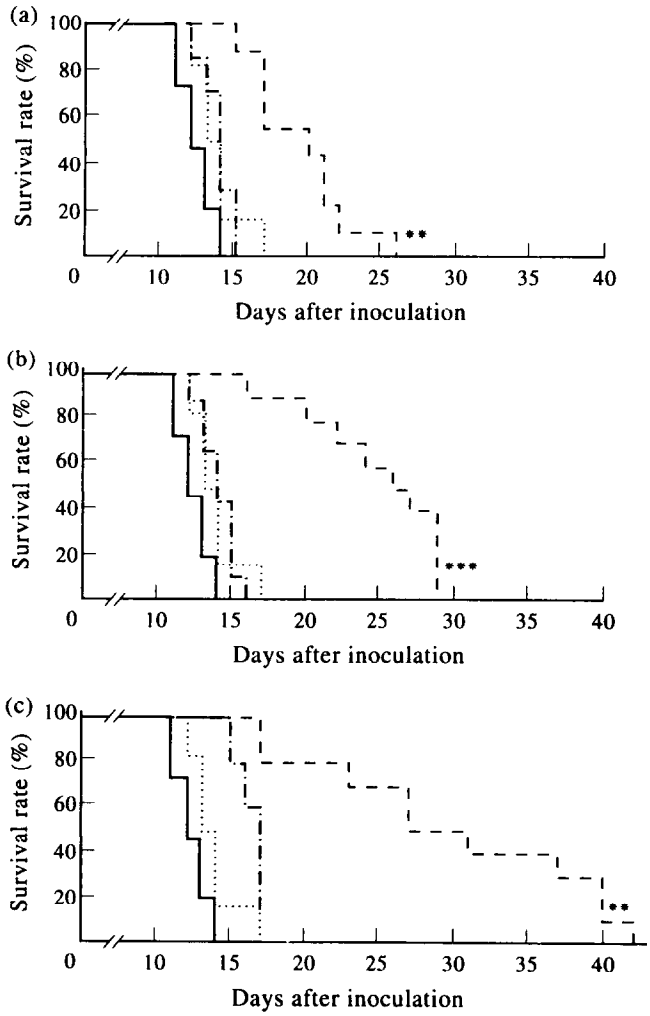

Figure 5. Therapeutic effect of combination treatment with CDDP plus AMB on mean survival time. Tumour-bearing nude mice were untreated (———) or were treated with AMB 2.0 mg/kg alone (.....). (a) CDDP 1.0 mg/kg with (----) and without AMB 2.0 mg/kg (··—·—). (b) CDDP 1.5 mg/kg with (----) and without AMB 2.0 mg/kg (—·—·—). (c) CDDP 2.0 mg/kg were administered with (----) and without AMB 2.0 mg/kg (·—·—). **Significantly different ($P < 0.01$) from the group treated with CDDP alone. ***Significantly different ($P < 0.001$) from the group treated with CDDP alone.

Accumulation studies of CDDP in tumour-bearing nude mice

Platinum accumulation in tumour tissue was measured to clarify the mechanism by which AMB enhances CDDP cytotoxicity, since AMB increased intracellular accumulation of CDDP in HRA cells *in vitro*. Platinum accumulation was 0.22 ± 0.067 μg/g wet weight tissue with 1.5 mg/kg of CDDP alone (Figure 6). AMB increased platinum accumulation to 0.30 ± 0.04, 0.38 ± 0.031 and 0.57 ± 0.069 at doses of 0.5, 1.0 and 2.0 mg/kg of AMB, respectively.

DISCUSSION

Several studies suggested that exposure of AMB to cells can significantly enhance cytotoxicity of drugs such as nitrosourea, actinomycin D, vincristine and doxorubicin [9–13]. Medoff and colleagues demonstrated that AMB (30 and 50 μg/ml) in combination with actinomycin D resulted in a significant decrease in the number of variable cells, and increased the amount of [^{3}H]actinomycin D incorporated in HeLa cells, resistant to actinomycin D up to 6-fold [20]. Krishan and colleagues reported that in doxorubicin-sensitive and -resistant P388 cells, co-incubation with AMB caused a marked increase in doxorubicin retention [21]. This increase was larger in cells resistant to doxorubicin than in sensitive cells. In contrast to observations by Medoff and Krishan, *in vitro* studies with human ovarian carcinoma cell line (COLO319) failed to demonstrate any increased cytotoxicity of doxorubicin and melphalan by AMB at a concentration of 2.5 μg/ml [22]. Our results demonstrated that CDDP cytotoxicity was enhanced by combination with AMB in a dose-dependent manner in HRA cells, and the possible mechanism of potentiation might be increased intracellular accumulation of CDDP by AMB.

Since CDDP is the most important cytotoxic agent in ovarian carcinoma and CDDP-based combination chemotherapy has been administered to advanced ovarian carcinoma patients, enhancement of CDDP cytotoxicity *in vitro* and *in vivo* has been sought. Buthionine sulphoximine (BSO), inhibitor of glutathione synthesis [23], and 3-aminobenzamide, an inhibitor of poly ADP ribosylation [24], were reported to enhance CDDP cytotoxicity. These agents, however, have met with limited success because their effectiveness differed between cell lines, and they were too toxic for *in vivo* use. Since phase I trials of AMB have been tested to enhance anticancer drugs, such as 1,3-*bis*(2-chloroethyl)-1-nitrosourea (BCNU), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), doxorubicin, cyclophosphamide and methotrexate [14–16, 25], we examined whether AMB could also enhance CDDP cytotoxicity. Intracellular accumulation of CDDP was increased significantly by treatment with low concentrations of AMB (Figure 3). No other agents have been shown to increase intracellular accumulation of CDDP like AMB. Since cell survival was related to the intracellular accumulation of CDDP [26], AMB may be useful in enhancing CDDP cytotoxicity. Indeed, HRA cells were sensitised to CDDP by low concentrations of AMB (Figure 2).

The combination of CDDP and AMB prolonged MST in tumour-bearing nude mice in a dose-dependent manner (Figures 4, 5). This is the first report showing enhancement of CDDP cytotoxicity by AMB *in vivo*, although there are several reports on enhancing cytotoxicity of other anticancer drugs. Higher doses of CDDP were enhanced more by AMB, indicating that higher doses of not only AMB but also CDDP resulted in longer MST (Figure 5). Unfortunately, several clinical trials of AMB failed to demonstrate the effectiveness of AMB, contrary to what would be expected from animal experiments. No potentiation of CCNU by AMB was observed in 10 patients with colorectal carcinoma and 5 with renal cell carcinoma [27]. In 37 patients with non-small cell lung carcinoma, randomised between a combination of doxorubicin, CCNU, hexamethyl melamine and methotrexate, and the same combination with AMB, a higher objective response rate was obtained with the latter regimen, but the difference was not statistically significant [28]. Since AMB is too toxic to administer at a high dose, patients received the following doses of AMB: 7.5 mg/m^2 on day 1; 15 mg/m^2 on day 2; 30 mg/m^2 on days 3 and 4, according to Presant and colleagues [16, 24, 27, 28]. Unsatisfactory results might be due to a low dose of AMB in patients with disseminated solid carcinoma.

On the basis of earlier studies, we examined the effectiveness of low dose AMB on CDDP cytotoxicity in nude mice with carcinomatosis peritonitis, because a direct effect of AMB on CDDP cytotoxicity was expected. We demonstrated that i.p. treatment with CDDP plus AMB at clinical doses could produce a significant extension of MST in nude mice with carcinomatosis peritonitis compared to CDDP alone. No side-effects, such as nephrotoxicity and myelosuppression, were observed in the

Table 1. Haematological and biochemical data

	Group (days after treatment)				
	Control	CDDP alone		CDDP and AMB	
	0	5	10	5	10
Platelet ($\times 10^4$ μl)	87 ± 9.0	112 ± 17	72 ± 3.8	111 ± 3.4	79 ± 12
Haemoglobin (g/dl)	14 ± 0.8	15 ± 0.6	15 ± 0.7	14 ± 0.8	15 ± 0.3
RBC (10^6/μl)	703 ± 48	717 ± 29	692 ± 29	709 ± 40	702 ± 26
WBC (10^3/μl)	8.8 ± 11	9.3 ± 1.5	10 ± 2.7	10 ± 1.9	11 ± 2.5
BUN (mg/dl)	20 ± 6.3	22 ± 3.0	21 ± 3.5	23 ± 4.0	20 ± 2.1
Creatinine (mg/dl)	0.41 ± 0.07	0.35 ± 0.05	0.35 ± 0.05	0.33 ± 0.05	0.36 ± 0.1
TP (g/dl)	5.2 ± 0.9	5.3 ± 0.1	5.3 ± 0.2	5.2 ± 0.3	5.6 ± 0.4
GOT (U/l)	138 ± 24	150 ± 20	151 ± 13	151 ± 30	147 ± 21
GPT (U/l)	48 ± 7.4	50 ± 3.1	50 ± 2.1	49 ± 2.9	49 ± 2.8

Haematological and biological examinations were performed 5 and 10 days after administration of CDDP 2.0 mg/kg with or without AMB 2.0 mg/kg four times every other day. Each experimental group contained four nude mice. RBC, red blood cells; WBC, white blood cells; BUN, blood urea nitrogen; TP, total protein; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase. Haematological and biochemical examinations were performed 5 and 10 days after administration of CDDP with or without AMB, four times every other day.

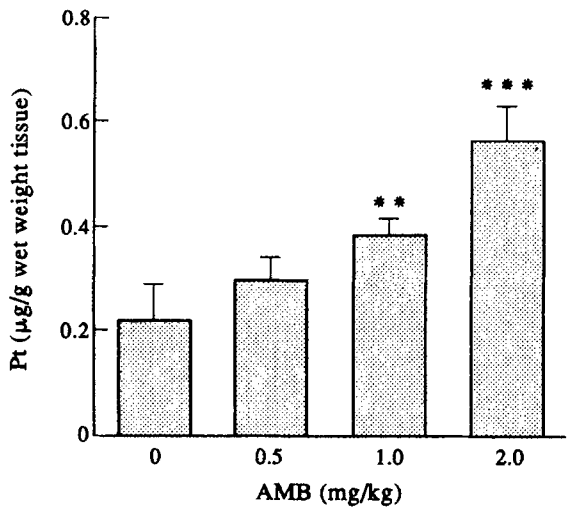

Figure 6. Platinum accumulation in tumour tissues in tumour-bearing nude mice after 2 h of combination treatment with CDDP 1.5 mg/kg and various doses of AMB. **Significantly different ($P < 0.01$) from the group treated with CDDP alone. ***Significantly different ($P < 0.001$) from the group treated with CDDP alone.

CDDP group, or CDDP and AMB group. Drug accumulation studies *in vivo* and *in vitro* demonstrated that the platinum accumulation in tumour tissue from mice treated i.p. with CDDP plus AMB was significantly increased compared to CDDP alone. These findings suggest that AMB could be a candidate for the treatment of advanced ovarian carcinoma in combination with CDDP.

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A Cross-national Investigation of Diet and Bladder Cancer

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The existence of a large unexplained portion of attributable risk, and the marked variation in bladder cancer rates globally, have stimulated an interest in the role of nutrition in cancer of the urinary bladder. For this cross-national comparison study, we had complete data available for 50 countries. Using stepwise regression followed by general linear modelling, age-truncated (45–74 years), world-standardised, sex-specific bladder cancer mortality rates were regressed on an array of nutritional and socioeconomic independent variables in an effort to identify important predictors of bladder cancer mortality. Separate principal components analyses were used to summarise the nutritional and the socioeconomic (SES) variables. In the stepwise analyses, using food scores expressed in kcal/day per capita (as opposed to the nutritional components), total fat consistently entered the model first, and explained the greatest share of variability (R^2) for both males and females. General linear models were fitted that included total fat, tobacco, alcohol, the three SES components (comprising seven socioeconomic predictors) and two food categories found significant in stepwise modelling, roots/tubers and vegetable oil. The R^2 values were 0.84 for male rates and 0.77 for female rates, meaning that these study factors account for 84% of bladder cancer mortality in men and 77% in women. Substitution of the nutritional components for the foods resulted in general linear models with slightly higher R^2 values (0.85 for males, 0.77 for females), but with attenuated fat effects. Results are discussed in light of biological plausibility.

Key words: bladder neoplasms, epidemiological studies, animal fat, economic factors
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INTRODUCTION

IN RECENT years, a profound interest in the role of diet and nutrition on cancers of several organ sites has emerged [1–3]. Evidence for an association between specific nutritional factors and cancer comes from a number of sources: ecological studies [4–6], analytic epidemiological studies [1, 2, 7], and laboratory animal experiments [2, 8, 9]. There is a widening view that bladder cancer may be linked with diet [10, 11].

The most important known risk factor for bladder cancer is tobacco smoking. It is estimated that approximately 25–50% of bladder cancers occurring in the U.S.A. can be attributed to smoking [12]. Other known risk factors, such as occupational exposure, can account for only a small additional fraction of bladder cancer cases. The large unexplained portion of attributable risk, as well as the marked variation in bladder cancer rates globally, argue for the examination of alternative environmental hypotheses, such as those related to diet [1, 12–14].

Therefore, in order to examine the role of nutritional factors in bladder cancer, a cross-national ecological study to identify dietary variables associated with male and female bladder cancer mortality rates was undertaken. We were motivated partly by