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Potentiation of *cis*-Diammine(1,1-cyclobutanedicarboxylato)platinum(II) by Amphotericin B in BALB/c Nude Mice Bearing Human Ovarian Carcinoma Cells

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Human ovarian carcinoma cells (HRA) were sensitized to *cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(II) (CBDCA) 1.2-, 2.1- and 3.4-fold by treatment with amphotericin B (AMB) at concentrations of 2.1, 5.4, and 10.8 μM, respectively. Moreover, the intracellular accumulation of platinum after 2-h exposure to CBDCA was increased significantly by AMB treatment. For estimating the enhancing effect of AMB on CBDCA cytotoxicity *in vivo*, we prepared HRA cell-inoculated nude mice. Ascites was evident 7 to 9 days after intraperitoneal (i.p.) inoculation of HRA cells, and the mice died of intraabdominal carcinomatosis 11 to 14 days (mean survival time (MST): 12.0±1.0 days) after inoculation. Treatment with AMB (2.0 mg/kg) alone increased the MST by only 1.2 days. Simultaneous treatment with CBDCA (12 or 15 mg/kg) and AMB (0.5 to 2.0 mg/kg) produced a significant increase in MST compared to treatment with CBDCA alone. Maximal MST (38.5 days) was obtained by treatment with 15 mg/kg CBDCA plus 2.0 mg/kg AMB, whereas the MST with 15 mg/kg CBDCA alone was 15.8 days. A drug accumulation study demonstrated that platinum accumulation in tumor tissues after i.p. treatment with CBDCA and AMB in tumor-bearing nude mice was increased significantly compared to treatment with CBDCA alone. These findings indicate that intraperitoneal combination chemotherapy with CBDCA and AMB is useful in nude mice with advanced ovarian carcinoma.

Key words: CBDCA — Ovarian carcinoma — Biochemical modulation — Amphotericin B — Intraperitoneal chemotherapy

Cis-diammine(1,1-cyclobutanedicarboxylato)platinum (II) (CBDCA), an analogue of *cis*-diamminedichloroplatinum(II) (CDDP), has appreciable activity against ovarian carcinoma.[1] It is less nephrotoxic, neurotoxic and emetogenic than CDDP.[1,2] Because of its favorable toxicity profile, CBDCA has been subjected to clinical studies.[3–6] However, development of resistance to CDDP or CBDCA often appears, and is one reason for chemotherapeutic failure. Although several approaches have been examined to enhance the cytotoxicity of anticancer drugs including platinum compounds,[7–9] these attempts have met with limited success because the modifying agents are too toxic to normal as well as tumor tissues.

Amphotericin B (AMB), a polyene antibiotic, is an important antifungal agent.[10] Earlier studies have suggested that binding of AMB to sterols in the cell membrane causes formation of 4 to 10 Å pores, resulting in alteration of membrane characteristics and loss of electrolytes and metabolites.[11,12] Several studies have suggested that treatment with AMB significantly enhances the cytotoxicity of drugs such as nitrosourea, actinomycin D, vincristine, adriamycin and CDDP.[13,14] A possible mechanism of action for the potentiation of cytotoxicity by AMB is an increase in cell permeability with a resulting increase in intracellular levels of cytotoxic agents.[13–15] Medoff *et al.*[16–18] indicated that AMB could potentiate the effect of cytotoxic agents in certain tissue culture systems in mice with AKR leukemia. These observations in animal models and in cell culture systems have led to examination of combinations of AMB and cytotoxic agents in Phase I and Phase II trials.[19–21] However, neither *in vivo* nor *in vitro* studies on the combination of AMB and CBDCA have been reported, although CBDCA is one of the most promising second-generation platinum compounds for treatment of ovarian carcinoma.

Recently the feasibility of intraperitoneal (i.p.) chemotherapy via a semipermanent i.p. catheter in patients with advanced ovarian carcinoma has been established and several studies have demonstrated that delivery of some anticancer drugs via the i.p. route is feasible and well tolerated, and that the peritoneal cavity is indeed exposed to higher drug concentrations than the rest of the body.[22] In view of the natural history of ovarian cancer, i.p. chemotherapy is an attractive way to increase the efficacy of primary chemotherapy,[3] or it may be used after first-line intravenous chemotherapy,[23] and salvage treatment of patients with small volume relapses appears to be the best situation to test i.p. treatment.[4,6,24] A pilot study of i.p. chemotherapy with CBDCA and interferon

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α in the treatment of relapsed ovarian cancer has been reported.[25]

On the basis of these studies, we examined the effectiveness of i.p. treatment with AMB in combination with CBDCA in nude mice with carcinomatosis peritonitis.

MATERIALS AND METHODS

Chemicals AMB, in the form of Fungizone, was obtained from Bristol-Myers Squibb Ltd. (USA) and was dispersed in a 5% dextrose solution immediately before use. CBDCA was obtained from Bristol-Myers Squibb Ltd. BCA protein assay kit was purchased from Pierce (USA). 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^{+}, namely bicinchoninic acid (BCA).

Nude mice Seven-week-old female BALB/c nude mice, each weighing 18–20 g, were purchased from Charles River Japan (Hino, Tokyo), and maintained in a pathogen-free environment. Mice were allowed food and water freely.

Cells HRA cells, derived from ascites of a patient with serous cystadenocarcinoma of the ovary, were kindly provided by Dr. Y. Kikuchi, National Defense Medical College, Tokyo. The cells were incubated in RPMI 1640 supplemented with 10% fetal bovine serum, 2 m*M* glutamine, 100 units/ml penicillin G, and 100 μg/ml 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-diphenyltetrazolium bromide (MTT) assay as described previously.[26] Two thousand cells per well were seeded into a 96-well microplate (Falcon 3072, Becton Dickinson, USA) and incubated continuously in various concentrations of drugs in a humidified atmosphere of 5% CO_2 at 37°C for 4 days. After the addition of 50 μl tetrazolium salt (MTT, Sigma, USA), culture was continued for 4 h at 37°C. The supernatant was then aspirated, and the resulting pigment was dissolved in dimethyl sulfoxide. The absorbancy at an optical density of 550 nm was measured by an 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.

Survival studies in nude mice For determining the effect of CBDCA *in vivo*, CBDCA was injected 3, 5, 7, and 9 days after the i.p. transplantation of 10^7 HRA cells in nude mice at 12 and 15 mg/kg. To examine the effect of AMB alone, 2 mg/kg AMB was injected i.p. in the same procedure. Furthermore, for determining the effect of AMB on CBDCA, both drugs were injected simultaneously in the same procedure (CBDCA 12 mg/kg plus AMB 0.5, 1.0, and 2.0 mg/kg; CBDCA 15 mg/kg plus AMB 0.5, 1.0, and 2.0 mg/kg). CBDCA, AMB, and the CBDCA plus AMB combination was well tolerated at these doses. Each experimental group contained 6 to 10 mice.

Drug accumulation studies *in vitro* Exponentially growing cells were incubated in freshly changed RPMI 1640 medium containing 10% fetal bovine serum and CBDCA with or without AMB at various concentrations for 2 h. Cells were washed twice with phosphate-buffered saline, collected by trypsinization and stored at −20°C. Intracellular platinum accumulation was measured by a modification of the method of Pera and Harder.[27] 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 assayed for platinum by flameless atomic absorption spectrophotometry (AA-8500 MK II, Nippon Jarrell-Ash Co. Ltd., Tokyo). The intracellular platinum accumulation was normalized to the cellular protein content estimated by a BCA protein assay kit.

Drug accumulation studies *in vivo* Drugs were injected i.p. simultaneously at doses of CBDCA 15.0 mg/kg, CBDCA 15.0 mg/kg and AMB 0.5, 1.0 or 2.0 mg/kg, 10 days after the i.p. transplantation of 10^7 HRA cells in nude mice. Mice were killed and tumor tissues were removed 2 h after administration. Tumor tissues were washed twice with phosphate-buffered saline and stored at −20°C. The platinum accumulation was measured by the atomic absorption method described above. Each experimental group contained 6 mice.

Statistical analysis Results are presented as the mean SD. The significance of differences in survival time was determined by using Wilcoxon's test and other statistical analysis was performed by using Student's *t* test.

RESULTS

Effect of AMB in HRA cells The cytotoxicity of AMB was investigated in HRA cells (Fig. 1). AMB alone had no antitumor effect on cell survival at concentrations below 5.4 μM, while higher concentrations were increasingly cytotoxic. The concentration of CBDCA alone required for 50% inhibition of growth (IC_{50}) was 22.6 μM, and treatment with AMB sensitized the HRA cells to CBDCA in a concentration-dependent manner (Fig. 2). The values of the dose modification factor (DMF), the ratio of IC_{50} for CBDCA to that to CBDCA with added AMB, were 1.4, 2.1, and 3.4 at the concentrations of 2.1, 5.4, and 10.8 μM AMB, respectively.

CBDCA accumulation studies in HRA cells Intracellular accumulation of CDDP in HRA cells was determined by atomic absorption spectrophotometry as described in "Materials and Methods." HRA cells were incubated with 400, 800 or 1200 μM CBDCA with or without 2.1, 5.4 or 10.8 μM AMB for 2 h. The intra-

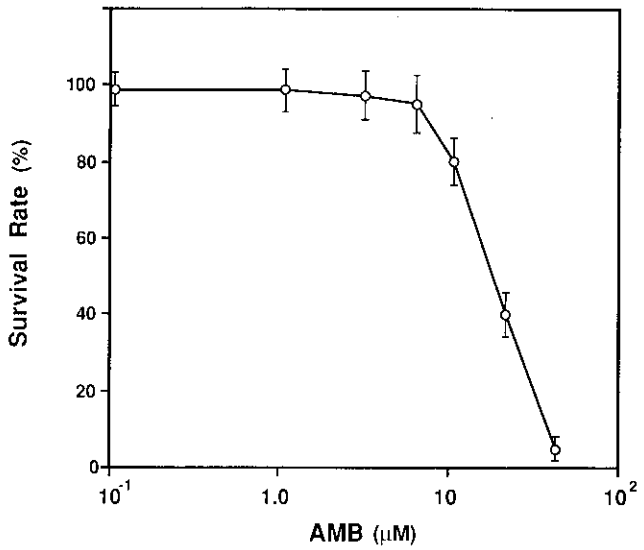

Fig. 1. Inhibition of cell growth by AMB as determined by MTT assay. Points are the mean values for 6 independent experiments. Bars indicate the SD.

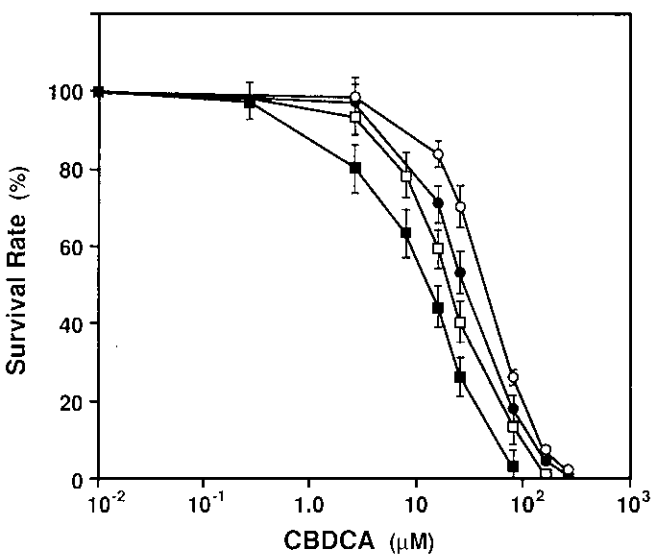

Fig. 2. Inhibition of cell growth by CBDCA with AMB. HRA cells were incubated with AMB 0 (○), 2.1 (●), 5.4 (□), and 10.8 μM (■). Points are the mean values for 6 independent experiments. Bars indicate the SD.

cellular platinum accumulation increased in a concentration-dependent manner and was significantly enhanced 1.3, 2.2, and 2.9 times by coincubation with 2.1, 5.4, and 10.8 μM AMB, respectively (Fig. 3). These findings suggested that the enhancing effect of AMB on CBDCA cytotoxicity was partially due to increased intracellular platinum accumulation.

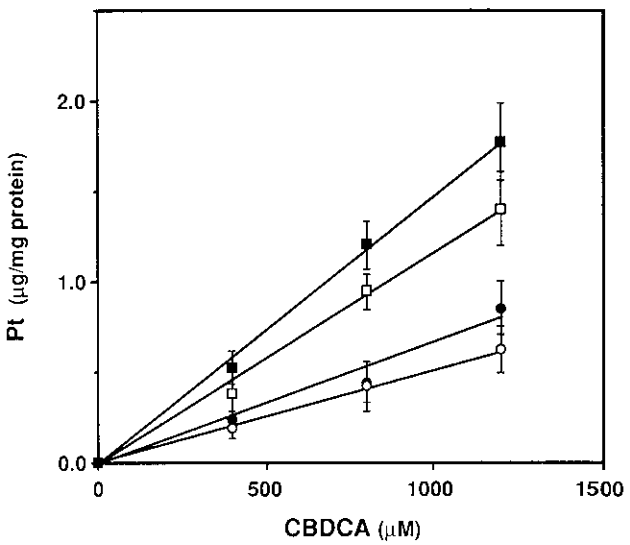

Fig. 3. Intracellular accumulation of CBDCA after 2-h exposure to various concentrations of CBDCA with AMB. HRA cells were incubated with AMB 0 (○), 2.1 (●), 5.4 (□), and 10.8 μM (■). Each point represents the mean of 4 independent experiments. Bars indicate the SD.

Effect of AMB and CBDCA on survival in nude mice

Fig. 4 shows the effects of CBDCA plus AMB on survival of BALB/c-nu/nu mice bearing HRA cells. Tumor-bearing mice given no drugs were used as the controls. Ascites was evident from 7 to 9 days after i.p. inoculation of 10^7 tumor cells, and the mice died of intraabdominal carcinomatosis from 11 to 14 days (mean survival time (MST): 12.0±1.0 days) after the inoculation. Treatment with 2.0 mg/kg of AMB had no antitumor effect (MST: 13.2±0.8 days). Treatment with 12 mg/kg of CBDCA extended the survival time for only 1.4 days (MST: 13.4±0.7 days), whereas simultaneous treatment with CBDCA plus AMB produced a significant, dose-dependent increase in survival time (0.5 mg/kg; MST: 14.9± 0.8 days, 1.0 mg/kg; MST: 21.5±10.3 days, 2.0 mg/kg; MST: 28.9±12.3 days) compared to CBDCA alone (Fig. 4A).

CBDCA alone produced a dose-dependent increase in survival time (Fig. 4B). However, the MST was extended by only 3.8 days (MST: 15.8±1.7 days) even by 15 mg/kg of CBDCA. Simultaneous treatment with CBDCA plus AMB produced a significant further increase in survival time (0.5 mg/kg; MST: 17.5±2.5 days, 1.0 mg/kg; MST: 25.8±11.6 days, 2.0 mg/kg; MST: 38.5±17.6 days) compared to CBDCA alone. AMB was more effective in combination with higher concentrations of CBDCA in tumor-bearing mice. No significant differences were found in the results of hematological and biochemical examinations between the CBDCA (15 mg/

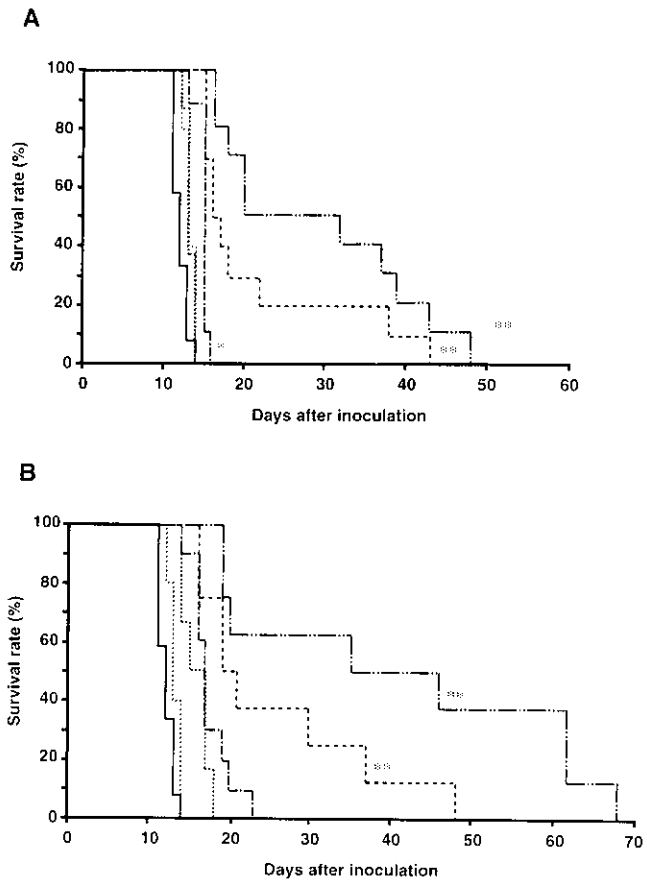

Fig. 4. Therapeutic effect of combination treatment with CBDCA plus AMB on MST. (A) Tumor-bearing nude mice were untreated (———), or treated with AMB 2.0 mg/kg alone (– – – –) or CBDCA 12 mg/kg alone (·····). Nude mice were treated with CBDCA 12 mg/kg plus AMB 0.5 (–·–·–), 1.0 (– – –), and 2.0 mg/kg (—·—). Data presented were from a representative experiment performed with more than 6 mice in each group. The significance of differences in survival was determined by using Wilcoxon's test. * Significantly different ($P<0.05$) from the group treated with CBDCA alone. ** Significantly different ($P<0.01$) from the group treated with CBDCA alone. (B) Tumor-bearing nude mice were untreated (———), or treated with AMB 2.0 mg/kg alone (– – – –) or CBDCA 15 mg/kg alone (·····). Nude mice were treated with CBDCA 15 mg/kg plus AMB 0.5 (–·–·–), 1.0 (– – –), and 2.0 mg/kg (—·—). The significance of differences in survival was determined by using Wilcoxon's test. ** Significantly different ($P<0.01$) from the group treated with CBDCA alone.

kg) and CBDCA (15 mg/kg) with AMB (2.0 mg/kg) group (data not shown).

Accumulation studies of CBDCA in tumor-bearing nude mice Platinum accumulation in tumor tissue was measured to clarify the mechanism by which AMB enhances

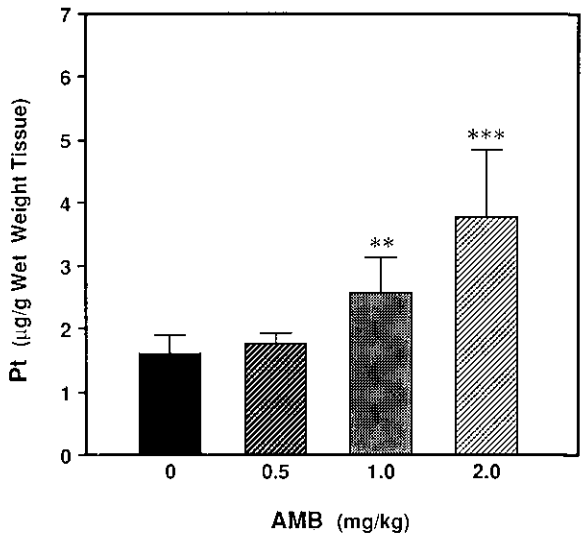

Fig. 5. Platinum accumulation in tumor tissues of tumor-bearing nude mice after 2-h combination treatment with CBDCA 15 mg/kg plus AMB 0.5, 1.0, and 2.0 mg/kg. The significance differences was determined by using Student's t test. ** Significantly different ($P<0.01$) from the group treated with CBDCA alone. *** Significantly different ($P<0.001$) from the group treated with CBDCA alone.

CBDCA cytotoxicity, since AMB increased the intracellular accumulation of CBDCA in HRA cells *in vitro* (Fig. 5). The amount of platinum accumulated was 1.61 $\pm$0.28 μg/g wet weight tissue with 15 mg/kg of CBDCA alone. On the other hand, AMB increased the platinum accumulation to 1.8$\pm$0.2, 2.6$\pm$0.6, and 3.8$\pm$1.2 μg/g wet weight tissue at the dose of 0.5, 1.0 and 2.0 mg/kg of AMB, respectively.

DISCUSSION

CDDP-based chemotherapy has been widely used on advanced ovarian caricnoma patients, and various methods to enhance the CDDP cytotoxicity *in vitro* and *in vivo* have been examined. Buthionine sulfoximine, an inhibitor of glutathione synthesis,[7] and 3-aminobenzamide, an inhibitor of poly ADP ribosylation,[8] have been reported to enhance the CDDP cytotoxicity. However, these agents are not clinically applicable because their effectiveness differs from cell line to cell line and they are too toxic for *in vivo* use. CBDCA, a second-generation CDDP analogue, is useful in the treatment of patients with ovarian carcinoma, since it is less nephrotoxic, neurotoxic, and emetogenic than the parent compound.[1,2]

AMB has been investigated *in vitro* as a possible enhancing agent of chemotheraputic drugs, since it may increase their intracellular levels. Medoff *et al.*[28] demon-

strated that AMB (30 and 50 μg/ml) in combination with actinomycin D resulted in a significant decrease in the number of viable cells and increased the cellular uptake of [^{3}H]actinomycin D in HeLa cells resistant to actinomycin D by up to 6-fold. AMB was able to increase cellular uptake of [^{14}C]nitrogen mustard in HT-29 human colon carcinoma cells and SKMES-1 human epidermoid carcinoma cells.[29] Krishan *et al.*[30] reported that in adriamycin-sensitive and resistant P388 cells, coincubation with AMB caused a marked increased in adriamycin retention. This increase was larger in cells resistant to adriamycin than in cells sensitive to it. In contrast to these observations, *in vitro* studies with the human ovarian carcinoma cell line (COLO319) failed to demonstrate any increased potentiation of adriamycin and melphalan cytotoxicity by AMB at a concentration of 2.5 μg/ml.[31] Thus, we examined whether AMB could enhance CBDCA cytotoxicity. Our findings demonstrated that the CBDCA cytotoxicity was enhanced by the combination with AMB in a concentration-dependent manner in HRA cells, and the mechanism involved appeared to be increased intracellular accumulation of CBDCA (Fig. 3). No other agent is known to increase the intracellular accumulation of CBDCA like AMB.

The combination of CBDCA and AMB prolonged the MST in HRA-cell-inoculated nude mice in a dose-dependent manner (Fig. 4). This is the first report on the enhancement of CBDCA cytotoxicity by AMB *in vitro* and *in vivo*, although there are several reports on the enhancement of the cytotoxicity of other anticancer drugs. The effects of higher doses of CBDCA were more enhanced by AMB, meaning that higher doses of not only AMB but also of CBDCA resulted in a longer MST. Unfortunately, several clinical trials of AMB failed to demonstrate the effectiveness of AMB, contrary to what had been expected from the animal experiments. The unsatisfactory results might be due to the low dose of AMB in patients with disseminated solid carcinoma. Since AMB is absorbed slowly via the peritoneum,[32] higher concentrations of AMB would be expected in the abdominal cavity with lower side effects if it were administered i.p. Moreover, the side effects of CBDCA are different from those of AMB, and no significant hematological or biochemical changes were observed 3 to 15 days after the treatment with CBDCA (15 mg/kg) and AMB (2.0 mg/kg)(data no shown). Our findings suggest that AMB could be a candidate for the treatment of advanced ovarian carcinoma patients in combination with CBDCA.

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