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Combination treatment of cis- and carboplatin in cancers restricted to the peritoneal cavity in the rat

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Abstract. In the present study, cisplatin (cDDP) and carboplatin (CBDCA) were combined in different in vitro and in vivo assays to determine whether combined cDDP and CBDCA treatment would eventually lead to a better antitumor response. Co-incubation of CC531 cells with cDDP and CBDCA led to higher intracellular Pt concentrations (30.5 ± 3.4 ng Pt/10^6 cells) than did cDDP (16.9 ± 9.4 ng Pt/10^6 cells) or CBDCA (1.28 ± 0.72 ng Pt/10^6 cells) incubation alone. In survival assays an additive cell kill was seen after combined treatment with cDDP and CBDCA. DNA binding experiments using isolated salmon-sperm DNA exposed to the drugs separately or in combination were in agreement with the survival studies (for cDDP a binding of 12.42 μg Pt/mg DNA; for CBDCA, 0.49 μg Pt/mg DNA; and for combined CBDCA and cDDP, 12.9 μg Pt/mg DNA at 76 h). Toxicity studies in rats treated with cDDP plus CBDCA required a dose reduction for cDDP amounting to 20% of the MTD, whereas the CBDCA dose could be maintained. Pharmacokinetics studies showed higher AUCs and $t_{1/2\beta}$ in plasma as well as the peritoneal cavity after combined treatment with cDDP and CBDCA (both given i.p.) or following cDDP given i.p. and CBDCA given i.v. Pt concentrations in peritoneal tumors corresponded with these observations, with higher Pt concentrations following combined treatment than after single-agent injection. In addition, combined administration of cDDP i.p. and CBDCA i.v. led to higher Pt concentrations in peritoneal tumors than did administration of both drugs i.p. (3.93 ± 0.9 vs 2.76 ± 0.2 mg Pt/g tissue). The higher Pt concentrations in the peritoneal tumors after combined treatment was associated with a significantly better antitumor response in comparison with that observed after single-agent treatment (a growth delay of 30.2 ± 5.6 days for cDDP i.p. plus CBDCA i.v. vs 16.1 ± 5.4 days for cDDP alone and 10.8 ± 4.2 days for CBDCA alone).

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Introduction

The human peritoneal cavity is a common site for tumor deposits at diagnosis of ovarian cancer and for tumor recurrence after initial "radical" surgical treatment of this tumor type as well as various gastrointestinal malignancies [18]. Because of the unusual natural course of ovarian cancer and low-grade gastrointestinal neoplasms, characterized by their tendency to remain confined to the peritoneal cavity, the control of metastatic disease in the peritoneal cavity is an important and challenging problem. Pharmacokinetic modeling for tumors confined to the peritoneal cavity has suggested that intracavitary administration of chemotherapeutic agents might result in a significantly greater drug concentration in the peritoneal cavity than in plasma [2, 7, 8].

In clinical studies, cisplatin (cDDP) has proved to be one of the most effective drugs available for the treatment of ovarian cancer [27, 28, 29, 31, 33, 38]. Intraperitoneal chemotherapy with cDDP was found to have an advantage over systemic cDDP chemotherapy [16, 25] in that complete remissions were achieved after i.p. cDDP chemotherapy in 30% of patients who suffered from ovarian cancer and who had earlier failed to respond to i.v. cDDP treatment [38]. This effect could be explained by the observation that the high concentrations of cDDP in the peritoneal cavity contribute to a large extent to the penetration of cDDP from the peritoneal cavity directly into the tumor, which leads to better intratumoral drug distribution and might result in better tumor responses [19, 20], although the latter possibility remains under investigation.

CDDP treatment can lead to severe side effects, including nephrotoxicity, neurotoxicity, and ototoxicity [43]. Less toxic analogues such as carboplatin (CBDCA) have recently been developed [13]. CBDCA has been shown to have activity against ovarian cancer similar to that reported for cDDP [4], with its dose-limiting toxicity being myelosuppression, including thrombocytopenia and leukopenia [4, 6, 37, 42]. Because of this favorable toxicity profile, CBDCA has been studied in a variety of tumor types [3, 14, 39, 44]. Prospective, randomized clinical trials have com-

pared single-agent CBDCA with cDDP in patients with advanced ovarian cancer, and the results demonstrated complete remission rates for CBDCA that were as high as those observed for cDDP [1, 35, 44].

Successful use of i.p. cDDP in cancers restricted to the peritoneal cavity and the promising use of i.v. CBDCA chemotherapy in ovarian cancer have led to trials of i.p. CBDCA aimed at improving the complete remission rate of ovarian cancer [5, 9, 23, 26]. Initial results of these studies indicated pharmacological advantages for CBDCA as compared with cDDP, such as slower elimination from the peritoneal cavity (because of its high stability and water solubility in comparison with cDDP) and lower protein binding [11, 26]. Tumor exposure to the drug via both the peritoneal cavity and the blood circulation after CBDCA treatment was enhanced as compared with that following i.p. cDDP administration [20]. In spite of these pharmacological advantages, the relative amount of CBDCA penetrating into peritoneal tumors and tumor cells was far lower than that observed for cDDP, resulting in lower Pt concentrations in peritoneal tumors [18, 20, 21].

Diminished access of chemotherapeutic agents to tumor cells and drug resistance will result in reduced efficacy [15]. Drug resistance is an obvious limiting factor in the effectiveness of chemotherapy of ovarian cancer, and retrospective analyses have demonstrated that the dose intensity of platinum compounds is a critical factor in achieving optimal results [32, 34]. Approaches to improve treatment should therefore be based on magnifying the extent of tumor exposure to platinum [30], which might be achieved through the combination of different platinum derivatives. Considering the favorable toxicity profile of CBDCA, combined treatment with cDDP and CBDCA seems logical. The nonoverlapping toxicities of cDDP and CBDCA may allow the total platinum dose to be increased, resulting in a better tumor exposure.

The objective of the present study was to investigate the possibility of combining cDDP and CBDCA treatment in cancers restricted to the peritoneal cavity of the rat in an attempt to improve the response rate.

Materials and methods

Animals and drugs. Male WAG/Rij rats were bred in the animal department of the Netherlands Cancer Institute. The animals were 8–12 weeks old and weighed 250–300 g at the time of their use. They were kept in a temperature-controlled room on a 12-h light/12-h dark schedule and were fed standard rat chow and tap water ad libitum. *cis*-Diamminedichloroplatinum(II) (cDDP) and *cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(ll) (CBDCA) were obtained as Platinol and Paraplatin (Bristol Myers, Weesp, The Netherlands). When necessary, dilutions were made in 0.9% sodium chloride immediately before each experiment.

Tumor model. The tumor (CC531) was a well-defined colonic adenocarcinoma induced by methylazoxymethanol with a doubling time in vitro of 16 h [21]. The tumor grows subcutaneously intraperitoneally, forming small tumor nodules (2–5 mm in diameter) on peritoneal surfaces and in vitro. In vitro, cells were cultured in 75-cm^2 flasks (Falcon, Oxnard, USA) in a humidified 5% CO_2 –95% air mixture and were replated twice a week at a density of 1×10^5 cells in fresh Dulbecco's modified Eagle's medium (DMEM; Irvine, Scotland) supplemented with 10% fetal calf serum (FCS; Gibco, The Netherlands) and 20 IU penicillin, 0.02 mg streptomycin, and 0.1 mg kanamycin/l. Single-cell suspensions for inoculation into animals were prepared by enzymatically detaching monolayer CC531 cells by adding 2 ml of 0.05% trypsin (Difco Laboratories, Detroit, USA) in 0.02% (w/v) ethylenediaminetetracetic acid (EDTA). Trypsin was subsequently inactivated by adding 10 ml of DMEM with 10% FCS. The cells were then centrifuged, resuspended, and diluted in phosphate-buffered saline (Oxoid, UK).

In vitro drug uptake in tumor cells. Each experiment was performed with 20×10^6 cells (3 flasks each containing about 7×10^6 cells). The cells were treated with either 5 µg cDDP/ml, 6.15 µg CBDCA/ml, or a combination of the drugs (5 µg cDDP/ml and 6.15 µg CBDCA/ml). Cells were incubated for 4 h at 37°C. After incubation, cells were washed with PBS, detached with trypsin, pooled, counted, and washed twice with PBS. Finally, the cells were suspended in 1 ml PBS and prepared for platinum determination [19].

Binding kinetics of CDDP and CBDCA to DNA. Isolated salmon-sperm DNA at a concentration of 1 mg/ml in phosphate buffer (0.1 *M* NaH_2PO_4) was incubated with either 0.5 m*M* cDDP, 0.5 m*M* CBDCA, or a combination of the drugs (0.5 m*M* cDDP and 0.5 m*M* CBDCA). At fixed time points, DNA samples were taken, purified from the free drug with the use of Sephadex G-50 gel filtration, and prepared for Pt determination by flameless atomic absorption spectroscopy (FAAS). The Pt-DNA adduct formation in CC531 cells was determined as described preciously [22, 40]. Briefly, CC531 cells were cultured on glass slides (2.6×6 cm) coated with ovalbumin (100 µl 0.5% ovalbumin/slide) and incubated with cDDP (25 µ*M*) or CBDCA (3 m*M*). Cells were fixed by successive incubations in cold (–20°C) 100% methanol (10 min) and acetone (2 min) and air-dried. The presence of platinated DNA was visualized by a double PAP staining. The nuclear staining intensity of individual nuclei was analyzed and quantified with a Knott (München, Germany) light-measuring device with a beam diameter of 5 µm, which was coupled to a Leitz Orthoplan microscope [40]. Data were analyzed by an Atari ST computer (Sunnyvale, USA) programmed with a version of the Histochemical Data Acquisition System (Hidacsys; Microscan, Leiden, The Netherlands). The integrated optical density of a selected area was expressed in arbitrary units. In each slide the nuclear staining density of 3–4 randomly selected areas, corresponding to 20–40 nuclei each, was measured [22].

Sensitivity of CC531 to CDDP and CBDCA. The sensitivity of CC531 cells to cDDP and CBDCA was tested by clonogenic assays. Cells in a single-cell suspension were plated in 6-well tissue-culture plates (Costar, Cambridge, UK) at 200 cells/well in a volume of 3 ml medium (DMEM). After 24 h incubation at 37°C, the cells were attached to the plates and a dilution of each drug or a dilution of the combined drugs was added. The ultimate concentration of cDDP ranged from 0.1 to 10 µg/ml and that of CBDCA, from 50 to 300 µg/ml. The cells were incubated for 1 h, after which they were washed two times with PBS and 3 ml of fresh medium was added. All plates were incubated at 37°C and 5% CO_2. Colonies containing more than 50 cells were scored at 7 days after cell plating. Colonies were counted and related to the control value.

Toxicity studies. Toxicity studies were performed to determine the MTD for combined i.p. cDDP and i.v. CBDCA treatment regimens. The doses of CBDCA and cDDP were 20 and 5 mg/kg, 25 and 5 mg/kg, 30 and 2.5 mg/kg, 30 and 3.25 mg/kg, and 30 and 4 mg/kg, respectively. At fixed time points for up to 52 days, blood samples were taken and creatinine, thrombocyte, leukocyte, and hemoglobin levels were determined.

Platinum concentrations in i.p. tumors after i.p. and i.v. chemotherapy. WAG/Rij rats were inoculated with 2×10^6 CC531 cells at day 0. At 4–5 weeks thereafter, small tumor nodules (2–5 mm in diameter) were present in 80%–100% of the rats. The tumors were situated on the diaphragm, peritoneum, and mesothelium. The rats were treated with cDDP (4 or 5 mg/kg given i.p. or i.v.) and/or CBDCA (30 mg/kg given i.v. or i.p.). The drugs were injected i.p. in a volume of 20 ml 0.9% NaCl and

i.v. in a volume of 1–2.5 ml (solution of 0.5 mg cDDP/ml and 1 mg CBDCA/ml). Tumor nodules were surgically removed from the peritoneal cavity at 24 h after treatment. Total platinum concentrations were determined by FAAS [19].

Pharmacokinetic studies. Plasma: Rats were anesthetized with ether and the carotid artery was cannulated. The cytostatic drugs were given i.v. in a volume of about 2 ml and/or i.p., dissolved (or diluted) in saline. At different time points after injection of the cytostatic drug, blood samples of about 0.75 ml were taken from the carotid artery. Clotting of the blood in the cannulas or samples was prevented by adding heparin. The Pt concentration in both plasma (total Pt) and ultrafiltrate (free Pt) was determined by FAAS after dilution in 0.2 *M* HCl/0.15 *M* NaCl. *Peritoneal cavity*: Rats were cannulated intraperitoneally by fixing cannulas in the left and right flanks of the peritoneal cavity. The rats were treated as described above, except that those treated i.v. only got an extra 20 ml of saline injected into the peritoneal cavity. Samples were prepared for determination by FAAS as described above. For total and free Pt the areas under the concentration-time curve (AUC) were determined, as were the maximal concentration (C_{max}) and the half-life time ($t_{1/2\beta}$).

Tumor growth delay. A tumor disk with a diameter of 2.5 mm and a thickness of 2.5 mm was implanted by hemoclip fixation on the inside of the ventral abdominal wall. At day 10 after implantation, the rats were treated with cDDP (3.5 mg/kg, i.p.), CBDCA (30 mg/kg, i.v.) or the two drugs combined (3.5 mg/kg cDDP and 30 mg/kg CBDCA given i.p./i.p. or i.p./i.v., respectively). For assessments of peritoneal tumor growth, new laparotomies were performed every 2 weeks. The size of the tumor was assessed by measuring three diameters of the tumor. The geometric mean of the three values was then calculated and used as the size estimate. The mean ± SD was then calculated from the geometric mean of tumors of each group. The growth delay of peritoneal tumors was defined as the time (in days) required for the tumors to regrow to a predetermined size (10 mm in diameter) in the treated group minus that required for the control group and was expressed as the mean value ± SEM.

Flameless atomic absorption spectroscopy. A model AA-40 atomic absorption spectrometer with a GTA 96 graphite tube atomizer (with Zeeman background correction) from Varian (Victoria, Australia) was used for Pt analysis. Pt concentrations were determined in plasma, peritoneal fluid, tumor and normal tissue, tumor cells, and Pt-bound DNA as previously described by Los et al. [19].

Statistical analysis. Student's t-test was usd to study significant differences, with P values of <0.05 being considered significant.

Results

Platinum uptake in cells

Platinum (Pt) uptake in CC531 cells was determined by incubating cells for 4 h (at 37°C) with either 5 μg cDDP/ml, 6.15 μg CBDCA/ml or cDDP and CBDCA combined (5 and 6.15 μg/ml, respectively). The results are shown in Fig. 1. Although the cells were treated with equimolar concentrations of cDDP and CBDCA, the cellular uptake of cDDP was about 13 times higher than that of CBDCA (16.9 ± 9.4 and 1.28 ± 0.72 ng Pt/10^6 cells, respectively). The cellular Pt concentration following simultaneous incubation with cDDP and CBDCA (30.5 ± 3.4 ng Pt/10^6 cells) was significantly higher than that of cells exposed to either cDDP or CBDCA or the sum of both drugs, which indicates at least an additive uptake of the drugs upon simultaneous incubation.

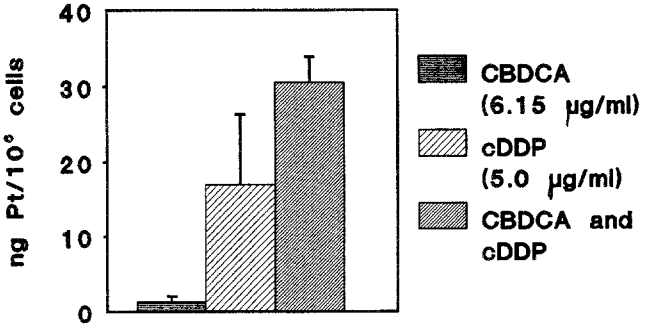

Fig. 1. Pt concentration in CC531 cells after incubation (4 h, 37°C) with CBDCA (6.15 μg/ml), cDDP (5 μg/ml), or both drugs combined (6.15 μg CBDCA/ml and 5 μg cDDP/ml). The bars represent the mean values ± SD for 5 experiments

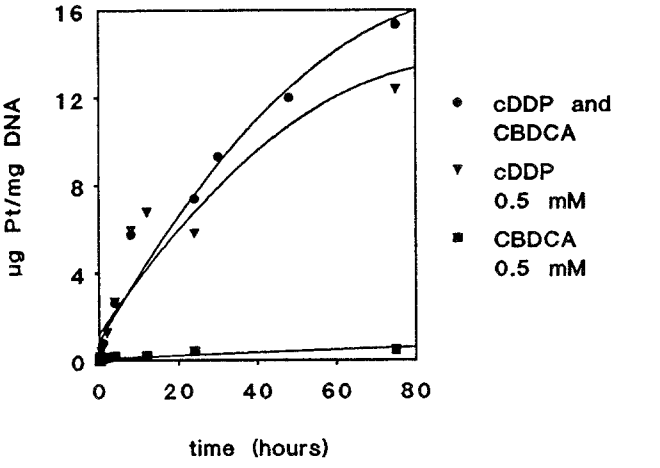

Fig. 2. Isolated salmon-sperm DNA (1 mg/ml) was incubated with CBDCA (0.5 m*M*), cDDP (0.5 m*M*), or CBDCA + cDDP. Samples were taken for up to 76 h, and the results represent the mean values for 2 experiments carried out in triplicate

Binding of CDDP and CBDCA to DNA

In solution, Pt binding to DNA was determined at fixed time points for up to 76 h. Isolated salmon-sperm DNA (1 mg/ml) was incubated with either 0.5 m*M* cDDP, 0.5 m*M* CBDCA, or a combination of cDDP and CBDCA (0.5 m*M* each) at 37°C. Total cDDP bound to DNA was about 25 times higher than the total binding of CBDCA to DNA (Fig. 2), while the binding of cDDP to DNA was much faster than that of CBDCA. Binding of Pt to DNA upon the administration of both drugs might be additive. However, since CBDCA in combination with cDDP contributes to only a small extent to DNA binding, it cannot be excluded that the DNA binding is lower than assumed and might even be similar to that following incubation with cDDP alone.

In CC531 cells, Pt-DNA adduct formation was determined after incubation with cDDP (25 μ*M*), CBDCA (3 m*M*), or a combination of the drugs (25 μ*M* cDDP/3 m*M* CBDCA). The levels of Pt-DNA adducts were similar after single-agent treatment with 25 μ*M* cDDP and 3 m*M* CBDCA (0.61 + 0.1 vs 0.56 ± 0.13 arbitrary units). Cells

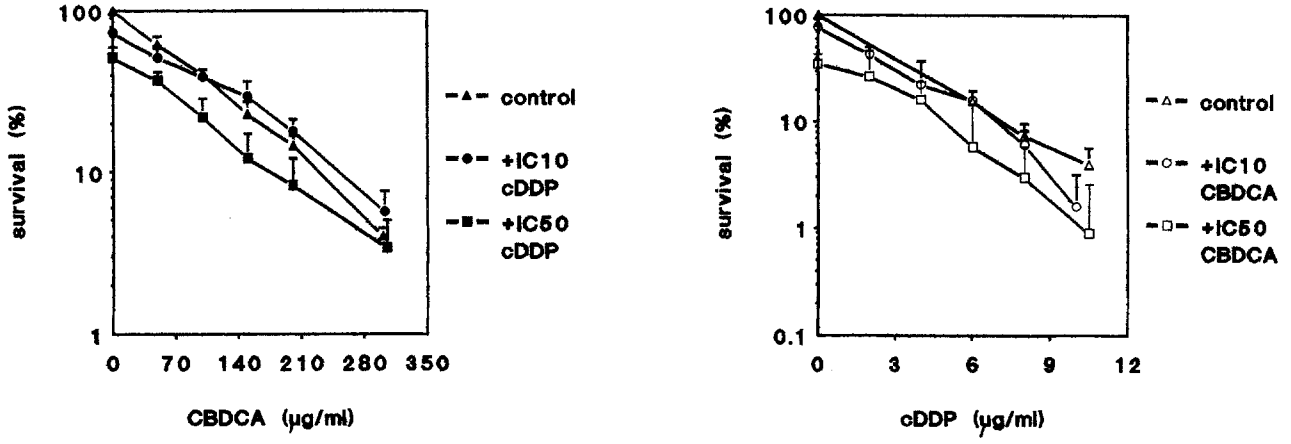

Fig. 3 A, B. Clonogenic assay of CC531 cells incubated with **A** CBDCA alone or in combination with the IC_{10} (0.5 µg/ml) or IC_{50} (2 µg/ml) of cDDP and **B** cDDP alone or in combination with the IC_{10} (10 µg/ml) or IC_{50} (50 µg/ml) of CBDCA. All curves were adjusted to the control values. Data represent the mean values ± SD of at least 5 wells.

Table 1. Pt concentrations in organs (in µg/g tissue) 24 h after CBDCA, cDDP, or combined CBDCA and cDDP administration in different treatment regimens

Treatment	Kidney	Liver	Lung	Spleen	Intestines
30 mg/kg CBDCA i.v.	12.6±2.2	2.5±1.5	2.1±0.5	2.7±0.7	3.1±2.1
30 mg/kg CBDCA i.p.	18.0±2.9	2.7±2.1	1.8±1.0	2.3±0.1	4.3±2.7
4 mg/kg cDDP i.v.	15.4±5.1	4.8±1.1	2.1±0.2	2.6±0.9	2.8±0.6
4 mg/kg cDDP i.p.	15.8±4.6	3.9±0.9	3.3±1.1	3.8±1.5	3.9±1.5
30 mg/kg CBDCA i.v. +4 mg/kg cDDP i.v.	15.2±4.8	6.5±0.8	2.9±0.6	4.8±0.8	10.0±2.8
30 mg/kg CBDCA i.v. +4 mg/kg cDDP i.p.	25.3±4.7	7.8±0.7	4.5±1.0	5.9±0.9	5.3±1.3
30 mg/kg CBDCA i.p. +4 mg/kg cDDP i.p.	20.0±8.4	7.6±2.3	5.0±0.8	5.4±1.1	5.9±3.2

Data represent mean values ± SD for at least 4 animals

treated simultaneously with 25 µ*M* cDDP and 3 m*M* CBDCA, however, showed no significant increase in Pt-DNA adduct levels (0.76 ± 0.23 arbitrary units).

Sensitvity to CDDP and CBDCA

The effect on the survival of cells incubated with cDDP (concentration range, 0–10 µg/ml), CBDCA (concentration range, 0–300 µg/ml) or a combination of the two drugs was determined by means of a clonogenic assay (Fig. 3). Cells were incubated for 1 h (at 37°C) and treated with CBDCA alone or combined with cDDP at an IC_{10} or IC_{50} dose (Fig. 3A) or with cDDP alone or combined with CBDCA at an IC_{10} or IC_{50} dose (Fig. 3B). The figures show that the initial percentage of cell kill is the result of an additive process, i.e., that simultaneous incubation at the IC_{10} or IC_{50} of cDDP or CBDCA with different CBDCA or cDDP concentrations, led to an extra 10% or 50% cell kill in comparison with the CBDCA or cDDP incubations alone. At higher concentrations, this additive effect disappears due to the relatively small effect of the IC_{10} or IC_{50} on these high incubation concentrations.

In vivo biodistribution of Pt

First, the MTD for the combination of cDDP and CBDCA was determined. Different schedules were tested by measuring creatinine, thrombocyte, leukocyte and hemoglobin levels. All rats treated with a standard dose of 5 mg/kg cDDP combined with different CBDCA concentrations (10, 20 and 25 mg/kg) died of renal insufficiency within 8 days. No toxic death was observed when the standard CBDCA dose (30 mg/kg) was combined with different cDDP concentrations (2.5, 3.25 and 4 mg/kg). Creatinine reached peak levels in blood of no more than 500 µ*M* and were back to normal after about 10 days, whereas thrombocyte counts reached normal values after 50 days, after reaching a nadir of approximately 250×10^9/l at around day 10. Leukocyte counts and hemoglobin reached normal values after about 50 days. The MTD of the combined treatment was therefore set at 4 mg/kg cDDP and 30 mg/kg CBDCA, which is a reduction of 20% in the cDDP dose in comparison to the MTDs for single-agent treatments.

To determine the Pt distribution in various tissues after the administreation of different treatment regimens, rats were treated with cDDP (4 mg/kg), CBDCA (30 mg/kg), or a combination of cDDP and CBDCA (4 and 30 mg/kg, respectively). The Pt uptake in the various organs is pre-

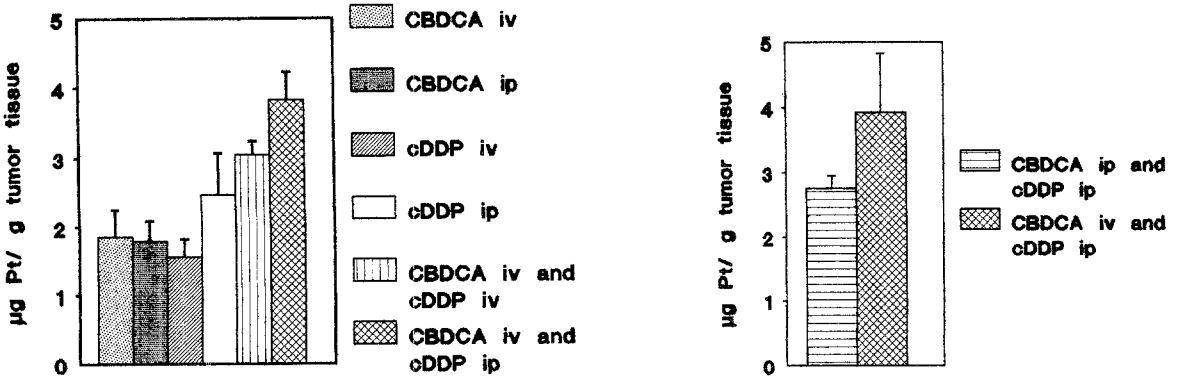

Fig. 4 A, B. Pt concentration in peritoneal tumors 24 h after treatment with **A** CBDCA given i.p. or i.v. (30 mg/kg),. cDDP given i.p. or i.v. (4 mg/kg), a combination of CBDCA (30 mg/kg) and cDDP (4 mg/kg), both given i.v., or CBDCA given i.v. and cDDP given i.p. and **B** CBDCA (30 mg/kg) and cDDP (4 mg/kg), both given i.p., or CBDCA (30 mg/kg), given i.v. and cDDP (4 mg/kg) given i.p.

Table 2. Pharmacokinetic data obtained following the administration of CBDCA (30 mg/kg, i. v.) or cDDP (4 mg/kg i. p.) to rats

	CBDCA i. v. (30 mg/kg)		cDDP i. p. (4 mg/kg)	
	Total Pt	Free Pt	Total Pt	Free Pt
AUC_{plasma} (0–24 h)	257 ± 63	96.3 ± 16	53.0 ± 1.2	22.9 ± 7.5
C_{max} (µ*M*)	324 ± 84	169 ± 5	11.1 ± 0.3	9.1 ± 2.8
$t_{1/2\beta}$ plasma) (h)	10.2 ± 1.3	9.7 ± 4.9	15.6 ± 2.1	4.6 ± 0.2
$AUC_{p.c.}$ (0–24 h)	750 ±233	669 ±177	302 ±33	356 ±69
$t_{1/2\beta\ p.c.}$ (h)	2.7 ± 0.3	2.2 ± 0.2	4.6 ± 1.5	2.7 ± 0.3

Data represent mean values ± SD for 3 animals

Table 3. Pharmacokinetic data obtained following treatment of rats with combinations of CBDCA (30 mg/kg i. p. or i. v.) and cDDP (4 mg/kg i. p.)

	CBDCA i. p. (30 mg/kg) + cDDP i. p. (4 mg/kg)		CBDCA i. v. (30 mg/kg) + cDDP i. p. (4 mg/kg)	
	Total Pt	Free Pt	Total Pt	Free Pt
AUC_{plasma} (0–24 h)	330 ± 71	216 ± 32	250 ±50	137 ± 16
C_{max} (µ*M*)	76 ± 19	93 ± 53	198 ±32	195 ± 19
$t_{1/2\beta\ plasma}$ (h)	8.1 ± 0.2	3.7 ± 0.3	12.6 ± 2.1	10.0 ± 2.0
$AUC_{p.c.}$ (0–24 h)	4162 ±460	3357 ±588	1291 ±93	1498 ± 130
$t_{1/2\beta\ p.c.}$ (h)	2.9 ± 0.4	2.4 ± 0.1	3.7 ± 0.1	1.9 ± 0.2

Data represent mean values ± SD for 3 animals

sented in Table 1. In most organs, combined exposure to cDDP and CBDCA resulted in an additive uptake of Pt. However, in the kidney and lung, following treatment with the combination of i.v. cDDP and i.v. CBDCA, the Pt concentration was significantly lower than the sum of the concentrations resulting from single-agent treatments (P = 0.011 and P = 0.016, respectively). No difference in the uptake of Pt in the various organs was seen between the i.p. cDDP/i.v. CBDCA group and the i.p. cDDP/i.p. CBDCA group.

Interestingly, there was no significant difference in the Pt concentration in tumor nodules between the i.p. and the i.v. CBDCA-treated groups (1.79 ± 0.3 and 1.85 ± 0.4 µg Pt/g tissue, respectively; Fig. 4A). However, the uptake of Pt following i.p. injection of cDDP was significantly higher than that following i.v. treatment (2.47 ± 0.6 vs 1.56 ± 0.3 µg Pt/g tissue, P = 0.032). When cDDP and CBDCA were given simultaneously, the combination of i.p. cDDP and i.v. CBDCA resulted in a significantly higher Pt concentration as compared with that following i.v. treatment with cDDP and CBDCA (4 and 30 mg/kg, respectively; 3.83 ± 0.4 vs 3.03 ± 0.2 µg Pt/g tissue, P = 0.022) or as compared with that following i.p. administration of both drugs (3.93 ± 0.9 and 2.76 ± 0.2 µg Pt/g tissue, P = 0.005; Fig. 4B).

Pharmacokinetics of CDDP and CBDCA

Pharmacokinetics studies were performed in rats treated with i.p. cDDP (4 mg/kg), i.v. CBDCA (30 mg/kg), or a combination of cDDP and CBDCA (4 mg/kg i.p. plus

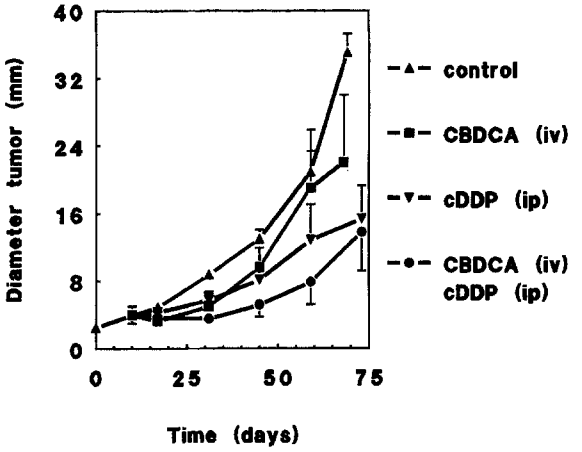

Fig. 5. Growth delay of peritoneal tumors after treatment with CBDCA (30 mg/kg, i.v.), cDDP (3.5 mg/kg i.p.), or CBDCA (i.v.) combined with cDDP (i.p.). Each point represents the mean value ± SD of the geometric mean of at least 3 tumors in each group

30 mg/kg CBDCA i.p. or 4 mg/kg i.p. plus 30 mg/kg i.v., respectively). At fixed time points for up to 24 h, blood or peritoneal fluid samples were collected and total and free Pt concentrations were determined (Tables 2, 3).

The pharmacokinetic data are shown in Tables 2 and 3. The area under the concentration-time curve (AUC) was related to the exposure of body tissues to Pt in the case of the AUC_{plasma} and to the exposure of tissues within the peritoneal cavity to Pt in the case of the $AUC_{p.c.}$. The AUC_{plasma} of total and free Pt in rats treated with i.v. CBDCA and i.p. cDDP was the additive result of the AUC_{plasma} of rats treated with CBDCA and cDDP alone. A similar additive process was seen for the C_{max} in plasma. There was no significant difference in the $t_{1/2\beta}$ between the CBDCA-treated group (i.v.) and the i.p. cDDP/i.v. CBDCA group, which could be explained by the observation that in the group treated with the combination, the clearance of Pt from the plasma mostly depended on the clearance of CBDCA. The $AUC_{p.c.}$ of free Pt in the i.p. cDDP/i.v. CBDCA group was significantly higher than the additive AUCs of the cDDP- and CBDCA-treated groups ($P = 0.003$). This finding could be explained by the lower $t_{1/2\beta}$ p.c. noted in the former group as compared with the rats treated with only cDDP or CBDCA, resulting in slower clearance from the peritoneal cavity. This effect would suggest some kind of equilibrium between Pt concentrations in plasma and peritoneal fluid. The difference between the AUC_{plasma} and the $AUC_{p.c.}$ of free CBDCA after i.v. CBDCA treatment might be explained by the relatively slow clearence of CBDCA from the peritoneal cavity after diffusion of CBDCA from the circulation into the peritoneal fluid (20 ml of saline given i.p. concomitantly with the i.v. administration of CBDCA). Since saline will clear faster than CBDCA from the peritoneal cavity, a relatively high CBDCA concentration will be detected in the peritoneal fluid, resulting in a high $AUC_{p.c.}$.

When both drugs were given i.p., the AUC_{plasma} of total and free Pt was significantly higher than that resulting from i.v. CBDCA/i.p. cDDP treatment ($P = 0.018$). The C_{max} and the $t_{1/2\beta}$ were significantly lower. The $AUC_{p.c.}$ of free Pt in the i.p. CBDCA/i.p. cDDP group was much higher than that in the i.v. CBDCA/i.p. cDDP group, which seemed to be based on that of CBDCA. Clearance of Pt from the peritoneal cavity was significantly slower when both drugs were given i.p. as compared with i.p. cDDP/i.v. CBDCA administration ($t_{1/2\beta}$). This effect might be due to the chemical properties of CBDCA, resulting in a slow clearance from the peritoneal cavity. From a pharmacokinetic point of view, it appears that the combined cDDP/CBDCA i.p. treatment leads to a better exposure of the tumor than does the combined i.v. CBDCA/i.p. cDDP treatment; however, this was not reflected in the Pt uptake experiments, where higher tumor Pt concentrations were found in the cDDP i.p./ i.v. CBDCA group.

Table 4. Growth delay of peritoneal tumors in rats

Drug	Route	Drug dose (mg/kg)	Growth Delay (days)
CBDCA	i.v.	30	10.5±4.2
cDDP	i.p.	3.5	16.1±5.4
cDDP+CBDCA	i.p. + i.v.	3.5 + 30	30.2±5.6

Rats were treated with CBDCA (i.v., 30 mg/kg; $n = 3$), cDDP (i.p., 3.5 mg/kg; $n = 15$), or a combination of CBDCA (i.v., 30 mg/kg) and cDDP (i.p., 3.5 mg/kg; $n = 3$). Growth delay was defined as the time (in days) required for tumors to regrow to a predetermined size (10 mm in diameter) in the treated group minus that required for the control group and was expressed as the mean value ± SEM

Tumor growth delay

The peritoneal tumor response was assessed after the administration of different treatment regimens (Fig. 5). Due to repeated laparotomies, which weakened the rats, the MTD for the combined treatment was reduced to 3.5 mg/kg cDDP and 30 mg/kg CBDCA. For a comparison of the effect of each treatment on the tumor response, the dose for the single treatments was set at 3.5 mg/kg i.p. cDDP and 30 mg/kg i.v. CBDCA. Tumor growth was delayed in all treated groups in comparison with the control. It appeared that combined treatment with i.v. CBDCA and i.p. cDDP, inhibited growth best. The tumor growth delay, as expressed in Table 4, shows a significant difference between the group treated with the combination and those given single-agent treatments (30.2 ± 5.6 vs 16.1 ± 5.4 and 10.5 ± 4.2 days for the combination-, cDDP-, and CBDCA-treated groups, respectively, $P<0.05$).

Discussion

In this study, cDDP and CBDCA were combined in different in vitro and in vivo models to determine whether combined cDDP/CBDCA treatment would eventually lead to better tumor exposure and responses. The two drugs have nonoverlapping toxicity profiles, with cDDP mainly causing nephro- and neurotoxicity, whereas the dose-limiting toxicity of CBDCA is myelosuppression [6, 9, 42, 43]. Combined treatment with these two drugs may allow an increase in the total Pt dose.

On treatment with equimolar concentrations, the uptake of Pt in CC531 cells was about 10–15 times higher for cDDP than for CBDCA. This finding is in agreement with earlier work [21]. An obvious explanation for the differing uptake of CBDCA and cDDP is the difference in their aqueous solubility (17 vs 1 mg/ml for CBDCA and cDDP, respectively [9, 20]. This chemical property negatively influences the passage of CBDCA through membranes [10, 20]. The difference in molecular weight (371 g/mol for CBDCA vs 300 g/mol for cDDP) seems to be of minor importance with regard to the uptake of the two drugs in cells [7]. Treatment with the combination of cDDP and CBDCA resulted in an uptake of Pt in the CC531 cells that was significantly higher than the additive concentrations following treatment with cDDP or CBDCA alone; therefore, synergism cannot be excluded. With regard to the latter caveat, Perez et al. [36] demonstrated recently that the interaction between platinum analogues could be synergistic, additive, or even antagonistic, depending on the cell line involved. Since nothing is known about possible interactions between cDDP and CBDCA and the data presented in this paper point in the direction of an additive effect for the combined treatment, synergism between the two drugs seems to be unlikely in this cell line.

cDDP bound to isolated salmon-sperm DNA much faster and to a greater extent than does CBDCA. After 76 h, 25 times more cDDP than CBDCA was bound to DNA. The explanation for this effect is given by Knox et al. [17], who strongly support the idea that aquation is the rate-limiting step in the reaction of both of these compounds with DNA (there is a 112-fold difference in the rates of the aquation reactions of these two drugs in favor of cDDP), that cDDP and CBDCA therefore differ only in the kinetics of their interaction with DNA, and that once they have bound to DNA the effects are similar [12, 17, 41]. In the present study, DNA was also incubated simultaneously with cDDP and CBDCA. The Pt binding with isolated salmon-sperm DNA after the administration of the combination increased in an additive fashion. The data obtained from the binding of cDDP or CBDCA with DNA were in agreement with the work of Knox et al. [17]. In contrast to the DNA binding in vitro, the cDDP/CBDCA-DNA adduct formation seen in CC531 cells did not differ significantly from that observed after single-agent treatment. The latter finding might be due to the high cDDP and CBDCA concentrations used for the detection of Pt-DNA adducts. This possibility is in agreement with the survival data obtained in vitro. At high drug concentrations (IC_{90} level), there was no significant difference in cell survival.

Pharmacokinetic theory provided a basis for the use of i.p. chemotherapy by predicting a long time-concentration gradient between the peritoneal cavity and plasma [2, 7]. Since the penetration of drugs from the peritoneal cavity into the tumor is restricted to a few millimeters, it is important that effective plasma concentrations are reached if tumor nodules larger than a few millimeters in diameter are present [19, 20, 25]. The pharmacokinetic data obtained in this study show that after combined treatment with cDDP and CBDCA, higher AUCs are seen in both plasma and the peritoneal cavity as compared with those following single-agent cDDP or CBDCA administration. These data correspond with the higher concentrations of Pt reached in the tumors after combined treatment as compared with those attained after single-agent cDDP or CBDCA treatment. Higher AUCs in plasma and the peritoneal cavity were achieved after the administration of i.p. cDDP/i.p. CBDCA in comparison with i.p. cDDP/i.v. CBDCA treatment. The high AUC observed for free Pt in plasma in the combined i.p. treated group is in agreement with earlier findings in which a relatively high AUC_{plasma} was found after i.p. administration of CBDCA [20]. From a pharmacokinetic point of view, this would suggest higher exposure of the tumor to Pt. However, the results of the Pt uptake in tumors show that the Pt concentration in the tumor was significantly higher when cDDP was given i.p. and CBDCA was given i.v. than after i.p. administration of both drugs. Since the AUC_{plasma} is similar for i.v. and i.p. CBDCA (Tables 2, 3), and the penetration of CBDCA from the peritoneal fluid into peritoneal tumors is negligible after i.p. treatment [20], the lower intratumoral drug concentrations observed after i.p. administration of CBDCA might be the result of lower peak plasma concentrations after the combined i.p. treatment. It has been suggested that CBDCA peak plasma concentrations are important in obtaining a high drug-concentration gradient in the interstitial tissue around the blood vessels [20]. Elferink et al. [10, 11] studied the pharmacokinetics of CBDCA in humans after i.p. and i.v. administration and demonstrated that the peak concentrations achieved in plasma following i.p. administration were approximately 4 times lower than those obtained after i.v. administration. Other pharmacokinetic parameters were comparable [10, 11]. Probably, also the combination of cDDP and CBDCA itself influences the uptake of the two drugs. Simultaneous uptake of cDDP and CBDCA into tumors may be different than would be expected from single-agent treatment. However, the influence of the interaction of cDDP and CBDCA on physiological and pharmacokinetic processes is as yet unknown.

Although cDDP and CBDCA show toxicity profiles that hardly overlap, combined treatment with cDDP and CBDCA may cause unacceptable toxicity. In earlier studies, the MTD for single-agent cDDP in the WAG/Rij rat was found to be 5 mg/kg [20]. In the present study, 5 mg/kg cDDP in combined treatment regimens with CBDCA doses, ranging from 10–30 mg/kg resulted in unacceptable nephrotoxicity, requiring a 20% dose reduction (to 4 mg/kg cDDP) to achieve tolerable toxicity. For CBDCA, the MTD is known to be 30 mg/kg [20] and in combined treatment with cDDP, this concentration needed no adjustment. These observations are in agreement with clinical findings in which a dose reduction was also necessary for cDDP but not for CBDCA when the two drugs were combined [32].

The higher tumor exposure achieved after treatment with a combination of cDDP and CBDCA, as measured by the AUC in plasma and the peritoneal cavity, was reflected not only in the tumor Pt concentrations; similar effects were observed in a variety of organs. The nephrotoxicity observed at these higher Pt levels after combined treatment with CBDCA (30 mg/kg) and cDDP (4 mg/kg) was acceptable. Since the nephrotoxicity seen after the combined treatment (i.v. CBDCA/i.p. cDDP) was com-

parable with that noted after single-agent i.p. cDDP treatment at the MTD [19], the higher Pt concentrations measured in the kidney after treatment with the combined regimen were probably attributable to CBDCA.

In the tumor-growth-delay experiments, all treatment groups showed a significant delay in tumor growth as compared with the nontreated group. Although no significant difference was observed within the different single-agent groups, there was a benefit for the combined i.p. cDDP/i.v. CBDCA administration. It was more effective than the single-agent treatments, which would be in agreement with the higher Pt concentrations found in the tumor. Preliminary data from clinical studies also demonstrate advantages for a combination of cDDP and CBDCA over single-agent cDDP or CBDCA [24]. Lund et al. [24] obtained a pathological complete response rate of 42% after combined treatment of patients with cDDP and CBDCA.

In conclusion, the in vitro studies, the tumor uptake experiments, and the pharmacokinetics studies indicate advantages for combined cDDP/CBDCA treatment. In addition, tumor growth-delay studies in the rat demonstrated an additive benefit for the combination, providing support for its clinical use. It is likely that diminished access to a tumor may be overcome by a high exposure. Definitive results of clinical studies [24, 32] with the combined administration of these drugs are therefore being awaited with great interest.

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