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CELLULAR PHARMACOKINETICS OF CARBOPLATIN AND CISPLATIN IN RELATION TO THEIR CYTOTOXIC ACTION

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Abstract—We have studied the cellular pharmacokinetics of carboplatin (CBDCA), as part of the evaluation of the antitumor activity of CBDCA in cancers limited to the peritoneal cavity in comparison with cisplatin (cDDP). The uptake of CBDCA into L1210 (lymphosarcoma), CC531 (colonic carcinoma), COV413.B (human ovarian carcinoma) and NB1 (human neuroblastoma) cells was 1.5 to 13 times lower than the uptake of cDDP. The uptake of CBDCA into human ovarian carcinoma cells, taken directly from patients, was also 8–20 times lower than cDDP. Platinum concentrations, expressed as a percentage of the total intracellular Pt concentration, were similar for CBDCA and cDDP in cytosol and nucleus/membrane fractions. A second major difference between the drugs was their binding to DNA. Less CBDCA–DNA than cDDP–DNA adducts were formed after incubation at equimolar amounts of drug with isolated salmon sperm DNA (5–25 times less). A 16–69 times higher concentration of CBDCA than cDDP was needed to induce similar changes in cell growth activity (50% [^{3}H]thymidine inhibition) in CC531 and COV413.B cells, indicating that equitoxicity can only be achieved when tumor cells are exposed to higher concentrations of CBDCA than cDDP. Similar toxicity was achieved in CC531 cells after incubation with a 16-fold higher CBDCA dose than cDDP. Comparable intracellular platinum concentrations, however, were obtained with a 10-fold higher CBDCA dose, suggesting that cellular pharmacokinetics of the drugs are different. Regarding drug uptake and pharmacokinetics the mechanism of action of CBDCA differed from cDDP at a cellular level.

Since its introduction into clinical trials in 1972, cisplatin (cDDP) has been found to be effective for the treatment of many types of cancer [1–6]. The side effects of cDDP treatment are however considerable, including nephrotoxicity, gastrointestinal toxicity and neurotoxicity [1, 7–9]. Second generation cDDP analogues have been synthesized in order to circumvent or reduce major dose limiting toxicities [10–14]. Prominent among them is carboplatin, a non-nephrotoxic analog that has shown promising antitumor activity in various Phase I and II studies [4, 15–18]. The reaction of cisplatin analogues with DNA has not been studied extensively, although carboplatin is assumed to from the same platinum adducts as cDDP [19, 20], based upon the structural identity of the intermediates [21].

Successful use of i.p. cDDP in cancers restricted to the peritoneal cavity and the promising use of i.v. carboplatin (CBDCA) chemotherapy in ovarian cancer, led to trials of i.p. CBDCA, hoping to improve the complete remission rate of ovarian cancer [22–24]. The first results of these studies indicated pharmacological advantages for CBDCA compared with cDDP, such as slower elimination from the peritoneal cavity and lower protein binding [22]. Taking into account the favourable toxicity profile, CBDCA may be a promising candidate for i.p. chemotherapy in cancers restricted to the peritoneal cavity. A major limitation of i.p. chemotherapy, however, is the ability of antineoplastic agents to penetrate peritoneal tumors [25, 26]. In view of this limitation it is certainly important to have a drug which enters tumor cells easily. Therefore, we have studied the cellular pharmacokinetics of CBDCA, as part of the evaluation of the antitumor activity of CBDCA in i.p. chemotherapy.

MATERIALS AND METHODS

Tumor cell lines. All tumor cell lines were grown *in vitro* and plated at a density of 1×10^5 cells/15 mL in fresh Dulbecco's Modified Medium (DMEM) with 10% foetal calf serum (Flow Laboratories). The cell lines used in the study were: L1210, a murine lymphosarcoma cell line; CC531, a colonic carcinoma; CC531.RL4, a cDDP resistant subline (about 16 times) derived from the CC531 colon carcinoma, selected by continuous incubation with increasing concentrations of cDDP up to 2 μg/mL; COV413.B, a human ovarian carcinoma cell line [27] (a gift of Dr P.I. Schrier, Leiden); COV413.B-PtR, a cDDP resistant human ovarian carcinoma cell line (about 8 times), obtained by continuous incubation of increasing cDDP concentrations up to 1 μg/mL [27] (a gift of Dr P. I. Schrier, Leiden);

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NB1, a human neuroblastoma cell line (a gift of Dr D. Kerr, Glasgow); and fresh isolated human ovarian carcinoma cells obtained from ascites of two patients. The CC531, the COV413.B and the NB1 tumor cell lines grow as monolayers, while the L1210 grows in suspension.

For the human ascites cells about 90% of the cells recovered were tumor cells, the remaining cells being white blood cells.

Drugs. cis-Diamminedichloroplatinum(II)(cDDP, Platinol^R) (Bristol Myers) and *cis*-diammine-(1,1-cyclobutanedicarboxylato)(II) (CBDCA, JM8) (Bristol Myers) were used in this study.

Tumor cell uptake of cDDP and CBDCA. Tumor cells ($20–40 \times 10^6$) were incubated with equimolar concentrations of cDDP (4.0 μg/mL) and CBDCA (4.92 μg/mL) for 1 or 4 hr at 37°. All incubations with Pt-drugs were performed while cells were in the log phase. After drug incubation, the cells were harvested, washed twice with PBS and prepared for Pt determination. Depending upon the experiment, cells were also incubated with equitoxic concentrations. Equitoxicity for CBDCA and cDDP for human ovarian carcinoma cells was estimated from patient pharmacokinetic data in which 5–7 times more CBDCA was given than cDDP [28].

Intracellular Pt *distribution.* CC531 and L1210 cells were incubated with equimolar concentrations of cDDP and CBDCA for 1 or 4 hr. After incubation, cells were harvested and washed twice with PBS. Treated tumor cells were disrupted by freezing and thawing (three times), put on a 0.25 M sucrose gradient and centrifuged at 1000 *g* for 10 min. Two fractions were collected, a nucleus/membrane fraction and a cytosol fraction. Both cellular fractions were prepared for Pt detection.

Binding kinetics of CBDCA and cDDP to DNA. Isolated salmon sperm DNA at a concentration of 2 mg/mL in phosphate buffer (0.1 M NaH_2PO_4, pH 7.0) was reacted with either cDDP (in 0.1 M NaH_2PO_4, pH 7, + 10% dimethyl sulfoxide) or CBDCA (in 0.1 M NaH_2PO_4,pH 7) to a final concentration of 1 mM and was incubated at 37° [21]. At fixed time points (0, 0.5, 1, 2, 4, 6, 24 and 50 hr) DNA samples were taken, purified from free drug with the use of Sephadex G-50 gel filtration and prepared for Pt determination.

Flameless Atomic Absorption Spectroscopy (FAAS). A model AA40 Atomic Absorption Spectrometer with a GTA 96 Graphite Tube Atomiser (with Zeeman background correction) of Varian (Victoria, Australia), was used for Pt analyses. Platinum concentrations were determined in whole cells, cell fractions and isolated DNA. Tumor cells ($20–40 \times 10^6$), tumor cell fractions or DNA were digested with 2.5 mL 65% HNO_3 at 170° in a PTFE digestion bomb for 2 hr. After cooling, the liquid was evaporated under a stream of air after addition of 5 mg NaCl. The residue was dissolved in 0.2 mL 0.2 M HCl containing 0.15 M NaCl. If necessary the tubes were placed in an ultrasonic bath for 10 min. All standards were treated in the same way as the samples, i.e. diluted with or digested in the appropriate matrix.

[^{3}H]*Thymidine incorporation.* Cells were trypsinized and plated in 6-well plates (5×10^5/well) in 3 mL DMEM. Cytostatic drugs were administered in triplicate after 24 hr (cDDP: 1.5, 3, 6 and 30 μg/mL; CBDCA: 3.7, 7.4, 18.5, 37.1, 74.2 and 111.3 μg/mL). The incubation period was 24 hr. [^{3}H]-Thymidine (3 μCi; sp. act. 25 μCi/mmol) was added to every well 23 hr after drug incubation was started. The [^{3}H]thymidine incorporation was terminated after 1 hr by adding 5 mL of 10% ice-cold TCA (trichloro-acetic acid) to each well. Cells were harvested, washed twice in 10% TCA and once in 50% ethanol and prepared for the detection of ^{3}H-radioactivity. All values were expressed as a percentage of controls.

Clonogenic assay. Drug sensitivity was determined using clonogenic assays in 6-well tissue culture plates. Cells were harvested with a trypsin (0.05% w/v) / EDTA (0.02% w/v) solution and counted. Single cell suspensions were plated into 6-well plates (200 cells/well) in a volume of 3 mL DMEM containing 10% foetal calf serum (FCS). cDDP and CBDCA were administered 24 hr later. After a 1 hr incubation period the plates were washed three times and fresh medium was added. After 7 days colonies greater than 50 cells were scored. All experiments were performed in triplicate.

Statistics. Analysis of variance was calculated by one-way ANOVA followed by Student's *t*-test, using the PC program Number Cruncher Statistical System (NCSS).

RESULTS

Pt *concentration in different cell lines after equimolar incubation with CBDCA and cDDP*

Approximately 2×10^7 cells of the following cell lines, CC531, CC531.RL4, L1210, NBI, COV413.B and COV413.B-PtR, were incubated at equimolar concentrations of CBDCA (4.92 μg/mL) and cDDP (4 μg/mL) for 1 hr. Table 1 shows the variation in uptake of cDDP and CBDCA in these cell lines. The highest uptake for both CBDCA (1.5 ± 1.0 ng/10^6 cells) and cDDP (11.0 ± 2.0 ng/10^6 cells) occurred in the NB1 cell line. The ratio between the uptake of cDDP and CBDCA ranged from 1.5 for the COV413.B-PtR cell line to 13.3 for the L1210 cell line.

For both cDDP and CBDCA the lowest uptake occurred in Pt resistant cell lines; in the COV413.B-PtR ovarian cancer cell line (0.3 ± 0.1 ng/10^6 cells) for cDDP and in the CC531.RL_4 colon carcinoma cell line (0.2 ± 0.1 ng/10^6 cells) for CBDCA. Prolongation of the incubation time had only a small effect on the ratio of cDDP to CBDCA uptake in the CC531 cell line (Table 1). After 1 hr incubation the ratio was 6.7 while after 4 hr incubation the ratio decreased to 5.8.

In vitro *uptake of cDDP and CBDCA into human ascites cells*

Human tumor cells obtained from peritoneal ascites of two patients with ovarian cancer were incubated with different concentrations of cDDP and CBDCA. As with the experimental cell lines, higher cellular Pt concentrations were seen after exposure to cDDP, both after 1 and 4 hr of incubation (ratio CDDP/CBDCA: 17.5 and 7.8, respectively,

Table 1. Platinum concentrations in various tumor cell lines after incubation with equimolar concentrations of cDDP* and CBDCA†

Cell lines	Incubation time (hr)	Pt concn (ng Pt/10^6 cells)‡		
		cDDP	CBDCA	Ratio cDDP/CBDCA
CC531	1	2.7 ± 1.6	0.4 ± 0.2	6.7
CC531	4	3.5 ± 1.6	0.6 ± 0.1	5.8
CC531.RL4	1	0.6 ± 0.1	0.2 ± 0.1	3.0
L1210	1	4.0 ± 1.0	0.3 ± 0.1	13.3
NB1	1	11.0 ± 2.0	1.5 ± 1.0	7.3
COV413.B	1	0.6 ± 0.2	0.2 ± 0.1	3.0
COV413.B-PtR	1	0.3 ± 0.1	0.2 ± 0.1	1.5

* 4 μg/mL cDDP.
† 4.92 μg/mL CBDCA.
‡ Values are the mean ± SD of at least triplicate measurements.

Table 2. Pt concentrations in human ovarian carcinoma cells after *in vitro* incubation with cDDP and CBDCA*

Patient	Drug	Incubation time (hr)	Drug concn (μg/mL)	Uptake (ng Pt/10^6 cells)	Ratio
I	cDDP	1	4	3.5	
	CBDCA	1	4.9	0.18	17.5
	cDDP	4	4	7.0	
	CBDCA	4	4.9	0.9	7.8
	cDDP	1	1.5	1.8	
	cDDP	4	1.5	3.5	
II	CBDCA	1	6.2	0.12	
	cDDP	1	5.0	3.1	31.0
	CBDCA	1	30.9	1.3	2.4

* Each determination was made in duplicate: the error between duplicate measurements did not exceed 5% of the mean.

in patient I and a ratio of 31 after 1 hr incubation period in patient II) (Table 2). Even a 6-fold increase in CBDCA concentration failed to achieve higher Pt concentrations than after cDDP (5 μg/mL). These data indicated that the uptake of CBDCA in human tumor cells was less than for cDDP, at least in these two patients.

Intracellular Pt *distribution*

The tumor cell lines CC531, COV413.B and NB1 were disrupted and fractionated into a cytosol and nucleus/membrane fraction after incubation with cDDP or CBDCA.

No difference in intracellular Pt distribution for cDDP and CBDCA could be detected in CC531, COV413.B and NB1 cells after a 1 hr incubation (Table 3). The nucleus/membrane fraction of the CC531 cells contained 26% of the total Pt concentration after cDDP and 23% after CBDCA treatment. About 56% of Pt was found in the nucleus/membrane fraction of COV413.B cells compared with 20% for NB1 cells. Incubation of CC531 cells with cDDP and CBDCA for 4 hr demonstrated that 35% of the total Pt uptake could be found in the nucleus/membrane fraction after cDDP treatment compared with only 14% after CBDCA (Table 3), indicating that, in spite of a similar distribution pattern after 1 hr, more Pt could be found in the nucleus/membrane fraction after cDDP treatment than CBDCA.

Binding of cDDP and CBDCA to isolated salmon sperm DNA

Isolated salmon sperm DNA was incubated with cDDP and CBDCA to determine the binding kinetics of both drugs with DNA. The binding rates were determined after incubation with equimolar concentrations of cDDP and CBDCA (1 mM). Cisplatin bound to DNA at an initial rate of 4.2 μM Pt/mg DNA/hr in contrast to CBDCA with a rate of 0.17 μM/mg DNA/hr. The binding of cDDP to DNA was also much faster, i.e. 12 μM Pt bound to 1 mg DNA in a 2 hr incubation period with cDDP treatment while a comparable DNA-Pt binding was only achieved with CBDCA after an incubation period of 25 hr (Fig. 1). The binding of cDDP with DNA was completed after 20 hr. At that time, almost

Table 3. Intracellular Pt distribution

Cell line	Time (hr)	Drug	% Pt in nucleus/membrane fraction*
CC531	1	cDDP	26 ± 11
		CBDCA	24 ± 7
COV413.B	1	cDDP	56 ± 9
		CBDCA	56 ± 7
NB1	1	cDDP	26 ± 6
		CBDCA	15 ± 10
CC531	4	cDDP	35 ± 3
		CBDCA	14 ± 1

* Values are the mean ± SD of at least three measurements.

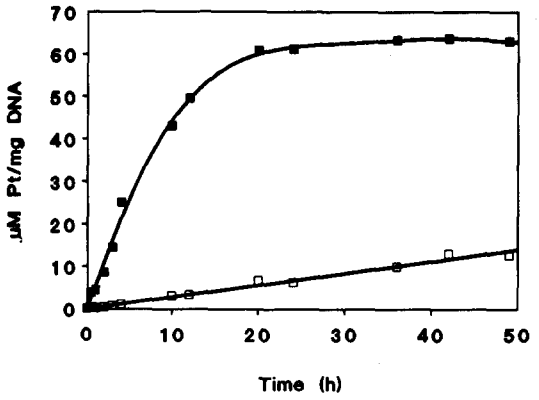

Fig. 1. Binding of cDDP and CBDCA to isolated salmon sperm DNA *in vitro*. (■) cDDP, (□) CBDCA.

10 times more cDDP was bound to DNA than CBDCA.

Inhibition of [^{3}H]*thymidine incorporation*

Changes in cell growth activity induced by cDDP and CBDCA in the CC531, CC531.RL$_4$, COV413.B and the COV413.B-PtR cell lines was determined by [^{3}H]thymidine incorporation. Higher CBDCA concentrations were required to induce the same inhibition of [^{3}H]thymidine incorporation in all four cell lines (Fig. 2). From these data, 50% inhibition was calculated for cDDP as well as for CBDCA (Table 4) and indicated that the different cell lines had to be incubated with 20–85 times more CBDCA than cDDP to evoke comparable DNA damage.

Correlation between Pt *concentration and toxicity in CC531 cells after incubation with cDDP and CBDCA*

Pt concentrations in CC531 cells were comparable for cDDP and CBDCA after incubation with 39.8 µg/mL and CBDCA and 4.0 µg/mL cDDP for 1 hr, demonstrating that a 10 times higher CBDCA dose in comparison with cDDP was required to achieve the same intracellular Pt concentration. No significant difference in cellular Pt concentration could be detected between equitoxic extracellular concentrations of cDDP and CBDCA after a 4 hr incubation period (Fig. 3) (one-way Anova test: α level > 0.05). The cellular Pt concentration of CC531 cells exposed to CBDCA was doubled, however, while during cDDP incubation the cellular content increased by a factor of 1.25 only.

The IC_{50} of CBDCA and cDDP for CC531 cells

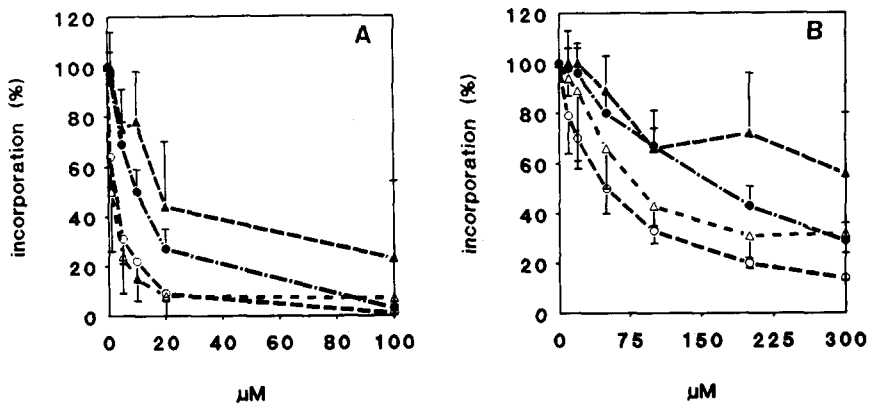

Fig. 2. Thymidine incorporation in CC531, CC531.RL4, COV413.B and COV413.B-PtR after incubation with cDDP (A) and CBDCA (B) for 24 hr. (△) CC531, (▲) CC531.RL4, (○) COV413.B, (●) COV413.B-PtR. Values are the mean ± SD of at least four experiments.

Table 4. Inhibition of the [^{3}H]thymidine incorporation *in vitro* after exposure to cDDP and CBDCA

Cell line	Drug concentration to induce 50% inhibition of the [^{3}H]thymidine incorporation*		
	cDDP† (μg/mL)	CBDCA† (μg/mL)	Ratio CBDCA/cDDP
CC531	0.36	30.8	85.5
CC531.RL4	6.0	124.6	20.7
COV413.B	0.84	17.8	21.2
COV413.B-PtR	3.0	61.5	20.5

* 50% inhibition data were obtained from Fig. 2.
† Incubation for 24 hr.

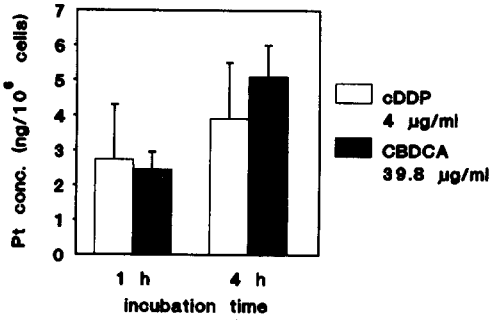

Fig. 3. Platinum concentrations in CC531 cells after incubation with cDDP and CBDCA.

was determined by clonogenic assays. To kill 50% of the cells after 1 hr incubation, 16 times more CBDCA than CDDP was needed (Table 5). The same difference was present after a 4 hr incubation period (factor 17.4) and declined to a factor of 5 after continuous incubation (0.05 μg/mL cDDP against 0.25 μg/mL CBDCA).

DISCUSSION

In this study we compared the uptake, the intracellular distribution and the binding to DNA of CBDCA with cDDP in several cell lines in order to evaluate the cytotoxic potential of CBDCA.

Data obtained from the present study demonstrated differences in the uptake of CBDCA and cDDP into cells. An obvious explanation is the difference in aqueous solubility (17 mg/mL vs 1 mg/mL for CBDCA and cDDP, respectively [28]) and maybe the difference in molecular weight (371 for CBDCA vs 300 for cDDP). The aqueous solubility probably most influences the passage of the drug through cell membranes, i.e. CBDCA will diffuse slower through cell membranes than cDDP. Howell *et al.* [15] and DeGregorio *et al.* [28] indeed demonstrated differences in the passage of both drugs across the peritoneal membrane. They reported a peritoneal clearance of 22 mL/min for cDDP and a peritoneal clearance of 7 mL/min for CBDCA, an approximate 3-fold difference. These data corresponded with those found by Elferink *et al.* [22]. The difference in uptake of both drugs after equimolar incubation *in vitro* varied from a factor of 1.5 for a cDDP resistant cell line, to a factor of 13 for the L1210 cell line. Increasing the incubation time for CC531 cells from 1 to 4 hr resulted in a slight decrease in the concentration ratio of cDDP/CBDCA. This was supported by results in human ascites cells. Higher Pt concentrations were detected after cDDP incubation (1 hr) in both patient I and II, while after a longer incubation period (4 hr) the ratio in Pt concentration vanished. Data obtained from patient II also suggested that an exposure of the tumor cells to a 6-fold higher CBDCA concentration, in comparison with cDDP, was still not sufficient to reach the comparable intracellular Pt concentrations. There was still a difference of a factor of 2.4. Nevertheless, the finding that the uptake of CBDCA in both cDDP resistant cell lines was less affected in comparison to the uptake of

Table 5. Effect of cDDP and CBDCA on the survival of CC531 cells

Incubation time	Drug concentration to reduce survival by 50%		
	cDDP (μg/mL)	CBDCA (μg/mL)	Ratio CBDCA/cDDP
1 hr	1.05 ± 0.2	17 ± 3.5	16.2
4 hr	0.57 ± 0.2	10 ± 2	17.5
Continuous	0.05 ± 0.01	0.25 ± 0.1	5

cDDP is most interesting. It indicates that CBDCA might be relatively more effective in situations of cDDP resistance *in vitro*.

It seemed that the drugs distributed in the same way after entering the cell. Similar percentages of CBDCA and cDDP were found in the cytosol and nucleus/membrane fractions. Differences occurred in the binding of the drugs to DNA, however, binding of CBDCA to isolated salmon sperm DNA was far less than for cDDP. These data are in agreement with the earlier studies of Micetich *et al.* [29], Knox *et al.* [21] and Terheggen *et al.* [30] in which 20–45 times more CBDCA was required to obtain equivalent DNA binding than after cDDP treatment. A detailed comparison of cDDP and CBDCA DNA binding indicated that these drugs differ only in their kinetics of the interaction with DNA. Hydration of CBDCA was 112-fold slower than cDDP and this difference corresponded to the different rates of binding to DNA [21].

Changes in cell growth activity evoked by CBDCA and cDDP were also studied. CBDCA is assumed to form the same type of Pt-DNA adducts and once bound to DNA, the subsequent effects were shown to be the same [21]. The data presented demonstrated that 16–69 times more CBDCA was required to induce the same inhibition of [^{3}H]thymidine incorporation. These data are in agreement with earlier studies in which 20–40-fold more CBDCA than cDDP was required to produce equivalent cytotoxicity in cells [21]. It is clear that CBDCA and cDDP undergo substitution reactions to form identical products but at significantly different hydration rates.

The question remains whether this kinetic difference is the determining factor in cell death. To answer this question the IC_{50} was determined for both CBDCA and cDDP on CC531 cells after 1 hr, 4 hr and continuous incubation. The data presented in Table 5 indicated that the activity of CBDCA did not increase in comparison with cDDP in the first 4 hr. Continuous incubation, however, diminished the difference between cDDP and CBDCA activity. The slower hydration reaction of CBDCA compared to cDDP may influence the binding rate relatively less during continuous incubation than after a 1 or 4 hr incubation period. In other words the relative increase in potency of CBDCA might partly explain the diminished CBDCA/cDDP ratio after continuous incubation. This is in agreement with studies of Curt *et al.* [31] and Roed *et al.* [32]. Nevertheless the 4 hr incubation period more closely simulates the human situation after i.p. treatment with CBDCA in terms of tumor exposure. Women with minimal residual disease ovarian cancer often have a 'dwell period' of 4 hr which means that CBDCA stays in the peritoneal cavity for 4 hr [23, 28]. The IC_{50} after a 4 hr incubation period indicated that 17 times more CBDCA was required than cDDP, while similar intracellular Pt concentrations were reached with a 10-fold higher CBDCA exposure than cDDP. This indicates that the cytotoxic action not only depended upon the Pt concentration in the cell but also on the binding to DNA. It seemed that differences in DNA binding kinetics also accounted for the cell death difference.

It is questionable whether CBDCA is the most suitable drug to administer i.p. Successful use of i.p. cDDP in cancers restricted to the peritoneal cavity and the promising use of CBDCA in i.v. chemotherapy, led to trials of i.p. CBDCA, in the hope of improving the complete remission rate of ovarian cancer [22–24]. Considering the fact that penetration of cDDP from the peritoneal cavity into the tumor is restricted to a few millimetres [33, 34], it is important to have a drug which enters cells readily. The data presented in this paper demonstrated, however, that the capacity of CBDCA to enter cells was less than that of cDDP, suggesting that CBDCA might be less effective in i.p. chemotherapy. The data in this paper indicated that doses 10–17 times higher than cDDP were required to achieve similar antitumor activity. Taking into account the clinical situation, in which only 5–7 times more CBDCA could be administered i.p., no increase in response rate could be expected after i.p. treatment with CBDCA.

In conclusion, the chemical properties of CBDCA in comparison with cDDP such as higher molecular weight, higher aqueous solubility and lower hydration rate strongly influenced its cellular pharmacokinetics. Firstly, the uptake of CBDCA was reduced in comparison with cDDP; and secondly, the binding to DNA was lower, due to the slower hydration reaction of CBDCA. It seemed that both the DNA binding and the cellular uptake were dependent on a limiting step. It is clear that the rate limiting step for the DNA binding is the hydration. Whether this is also the case for the cellular uptake is questionable. However, if the hydration also influences the cellular uptake it would explain the fact that the cellular distribution is the same for both drugs. The data in this paper therefore suggests that i.p. CBDCA treatment will probably be less effective in cancers restricted to the peritoneal cavity than treatment with cDDP [35]. Studies to confirm this are in progress.

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