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Comparative Pharmacological, Toxicological and Antitumoral Evaluation of Free and Liposome-encapsulated Cisplatin in Rodents

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The systemic toxicity and efficacy of cisplatin (CDDP) were examined *in vitro* and *in vivo*. Procedures were performed before and after the antineoplastic agent was encapsulated into multilamellar liposomes (L-CDDP). *In vitro* cytotoxicity evaluation in L1210 murine leukaemia and NIH OVCAR human ovarian cancer cells showed IC_{50} values of 0.14 and 0.05 μg/ml with CDDP or L-CDDP, respectively. *In vivo*, mice injected intravenously with L-CDDP had plasma levels of platinum 4-fold higher than with CDDP. The $t_{\frac{1}{2}}\alpha$ was 2 h and the $t_{\frac{1}{2}}\beta$ exceeded 48 h with L-CDDP; whereas a $t_{\frac{1}{2}}\alpha$ of 15 min and $t_{\frac{1}{2}}\beta$ of 12 h was observed with CDDP. The values of platinum in liver, spleen, kidneys, lungs and heart were substantially higher in L-CDDP-treated compared to CDDP-treated mice. Cytotoxic evaluation of both agents was tested *in vitro* (murine L1210 leukaemia and NIH OVCAR cell line) and *in vivo* (male CD_2F_1 mice). CDDP and L-CDDP showed similar cytotoxicity in tissue culture. At the highest dose given, 12 mg/kg intraperitoneally (i.p.), L-CDDP showed higher antitumour efficacy demonstrated by an increased life span of the mice. The CDDP treatment at the highest dose was lethal to all the tumour bearing mice. The nephrotoxicity in rats (blood urea nitrogen and creatinine evaluation) of L-CDDP administered i.p. was significantly less than with CDDP. In addition, the ability of kidney slices to transport organic anions [paraaminohippurate (PAH)] and consume O_2 was substantially decreased in rats treated with free CDDP compared to L-CDDP. Accordingly, the liposomal encapsulation of CDDP attenuates its nephrotoxicity, but allows maintenance of antitumour efficacy and may be a potentially effective modality in clinical settings.
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INTRODUCTION

CISPLATIN (CDDP) is one of the most widely used antitumour agents in the chemotherapeutic treatment of testicular, ovarian and bladder cancers [1–4]. The major dose-limiting adverse effect of CDDP is renal toxicity; other side-effects are of gastrointestinal, haematological, and neurological origins [5]. Furthermore, some tumours are totally refractory to CDDP [6]. These factors necessitate the synthesis and evaluation of new platinum compounds [7–10].

Liposomal encapsulation reduces the toxicity associated with many antitumour agents by altering their pharmacokinetics and disposition [11–13]. It has been demonstrated that liposomal encapsulation of other chemotherapeutic agents attenuates the well recognised dose-dependent toxicity, which is due, at least in part, to reduced uptake of liposomal-entrapped drug into tissue. This has been found both in animal models [14, 15] and in human studies [16]. Deliconstantinos *et al.* [17] showed that in an *in vivo* murine model, a liposomal-encapsulated lipophilic analogue of CDDP (L-CDDP) was specifically concentrated in an ADJ/PC 6A murine tumour. Yatvin *et al.* [18] found that temperature-sensitive liposomes with entrapped CDDP were active against mouse sarcoma 180. However, Steerenberg *et al.* [19] needed twice the amount of L-CDDP injected intravenously (i.v.) into rats bearing a LOU/M IgM immunocytoma to achieve antitumour activity equal to free CDDP. Nevertheless, they demonstrated reduced nephrotoxicity from L-CDDP compared to CDDP. In the current study, we report a preparation of liposomal-encapsulated cisplatin (L-CDDP) that has a higher total drug encapsulation, demonstrates higher therapeutic efficacy when administered intraperitoneally (i.p.) and maintains reduced nephrotoxicity. In addition, we assess the metabolic parameters related to the reduced renal damage with L-CDDP compared to free L-CDDP.

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MATERIALS AND METHODS

Reagents

Cisplatin was kindly provided by the Developmental Therapeutic Program of the National Cancer Institute. Phosphatidylcholine, phosphatidylserine, cholesterol, blood urea nitrogen (BUN) and creatinine assay kits were purchased from the Sigma Chemical Co. (St. Louis, Missouri). Mannitol (20% Osmitrol) was purchased from Travenol Laboratories, Inc. (Dearfield, Illinois). All other chemicals were reagent grade.

Animals

Male CD_2F_1 mice and female DBA/2 mice, 8–10 weeks old, 20 – 25 g; and male Sprague–Dawley rats, 11–13 weeks old, 175–200 g were purchased from Charles River Breeding Laboratories, (Boston, Massachusetts). All the animals were maintained according to accredited procedures at the Georgetown University animal facility, and fed Purina chow and water *ad libitum*.

Cell cultures

The murine ascitic L1210 cell line was obtained from the Animal Genetics Branch, Developmental Therapeutic Program,

Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland. The L1210 leukaemia was maintained by serial intraperitoneal passage in female DBA/2 mice. NIH OVCAR cells, human ovarian cancer, obtained from ATCC (Rockville, Maryland) were grown as monolayer in 25 cm^3 plastic Falcon flasks in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum and gentamicin (50 μg/ml).

Liposomal preparation

Phosphatidylcholine, phosphatidylserine and cholesterol in a 3:3.1:3.7 molar ratio were dissolved in chloroform: methanol 5:1 (v/v) and dried in a rotating flask under vacuum. Cisplatin crystals, 3 mg/ml, were hydrated in a buffer solution containing 0.25% NaCl and 5% mannitol for 1 h at room temperature and homogenised continuously for 15 min on a bench sonicator to make a 3 mg/ml solution of cisplatin. This solution of cisplatin was added to the dried lipids film, dispersed by sonication (Mettler Electronics) for 10 min, purged with nitrogen and left overnight in a ground neck flask for hydration. The hydrated suspension was sonicated for 20 min at 37°C at Output Control 7 in a Cuphorn sonicator (Model #380 Heat Systems Ultrasonics, Farmingdale, New York) to form liposomes. The liposomes were dialysed in 0.9% saline for 3 h at room temperature. The dialysis fluid was changed every hour for a total of three changes. The platinum content of the liposomes was measured by atomic absorption spectrophotometry. The free cisplatin and the liposomal-encapsulated cisplatin were adjusted in normal saline for injections at 2% (0.2 ml/10 g) of the body weight of the mice.

Cytotoxicity evaluations

L1210 (1×10^6 viable log phase cells) were cultured in 25 cm^3 plastic flasks, in 10 ml RPMI medium supplemented with various doses of either CDDP or L-CDDP. At 24, 48 and 72 h, L1210 cells were harvested by centrifugation (2000 *g*, 10 min., 25°C), washed with fresh medium without drugs, and the number of viable cells counted on a haemocytometer by the Trypan blue dye exclusion method. Control cultures were L1210 cells maintained in RPMI-1640 without CDDP or L-CDDP. NIH-OVCAR cells were grown in monolayer in RPMI medium. Cells were harvested at log phase and plated in six-well Falcon Petri dishes at 500 viable cells/well, 3 ml medium per well. These cultures were grown for 24 h for attachment and thereafter treated with various doses of CDDP or L-CDDP. Control cultures were treated either with saline or 'empty' liposomes (liposomes without CDDP). After 24, 48 and 72 h posttreatment, both CDDP- and L-CDDP-treated cultures were washed with fresh medium to remove the drugs. The cultures were left with complete media for 10–12 days and resulting colonies were fixed and stained with methylene blue (0.5% carbol fuchsin and 0.13% methanol) solution and were counted under a microscope.

Treatment of animals

For *in vivo* antitumour studies, male CD_2F_1 mice were injected i.p. on day 0 with 1×10^5 viable L1210 leukaemia cells. On day 1 they were randomly divided into groups of eight mice. Each group received various doses of either CDDP or L-CDDP. CDDP and L-CDDP were administered i.v. via the tail vein and/or i.p. on days 1, 5 and 9, and in another group on days 1 and 9. Control groups consisted of mice given saline or empty liposomes i.v. and/or i.p. The injection volume was 2% body weight (0.2 ml/10 g). Survival was recorded every 24 h. Mice were evaluated until day 40. Data are expressed as:

$$\frac{\text{median survival time of treated mice} \times 100}{\text{median survival time of control mice}}.$$

For pharmacological studies, male CD_2F_1 mice were injected i.v. via the tail vein with either CDDP or L-CDDP (6 mg/kg body weight). At specific points in time blood from four mice per group was obtained by retro-orbital exsanguinations after the induction of anaesthesia. The blood was collected into heparinised tubes (10 U/ml) and centrifuged at 1500 *g* for 10 min at 4°C to isolate the plasma. The liver, kidney, spleen, lung and heart were collected. The plasma and organs were frozen at −20°C until the platinum could be measured.

For toxicological studies, male Sprague–Dawley rats (five in each group) were treated i.p. with CDDP at a dose of 3 mg/kg on days 1 and 5 and with L-CDDP at doses of 3 and 4.5 mg/kg on the same days. Control rats received saline or empty liposomes. Rats were observed daily and weighed. At day 9, all the rats were anesthetised with pentobarbital. Before killing blood samples were collected from the abdominal aorta for BUN and creatinine analysis. The whole kidneys were excised, weighed and finally processed for evaluation of nephrotoxicity.

To assess renal function, [^{3}H]paraaminohippurate (PAH) and [^{14}C]tetraethylammonium (TEA) uptake and QO_2 consumption were followed by incubating rat renal slices. The methods used to assess organic ion uptake by renal slices have been described [20,21]. To quantify *in vitro* accumulation of organic ions in renal slices, an S/M ratio was used as described by Goldin *et al.* [22]. This is the ratio (found after incubation) of the amount of isotopic PAH and TEA which has accumulated in the slice (S) estimated by disintegration per min in 1 g compared to the amount of isotopic PAH and TEA in the medium (M) estimated by disintegration per min in 1 ml of medium.

Oxygen consumption was assessed with a Yellow Springs Biological Oxygen Monitor using a Clark Electrode [23]. The medium was modified Krebs phosphate-buffered solution [24, 25]. Substrate was 2 mmol/l glutamine. Results are expressed as μl of oxygen per mg tissue consumed per min.

Platinum analysis

To date, attempts to determine platinum levels in serum and organ samples by flameless atomic absorption spectrophotometry, without prior lengthy tissue processing, have been unsuccessful [26]. We developed a more efficient and reproducible method of accurately measuring small quantities of platinum both in plasma and tissue samples. This method bypasses the lengthy platinum extraction procedures and tissue processing prior to platinum measurements. This was facilitated by the buffer system utilised and the incubation procedure used to homogenise the tissue samples to burn evenly without splattering in the graphite furnace.

Tissues were homogenised (Brinkman Instruments, Westbury, New York) with 10 volumes of 0.1% Triton X100 in 0.1 mol/l HCl. Tissue samples were heated for 1 h at 90°C followed by incubation at 37°C overnight in screwcapped glass tubes. After digestion, the tissue suspensions (20 μl) were injected straight into the furnace for the platinum estimations. To adjust for matrix effects, control plasma and tissue homogenates were spiked with cisplatin and measured for platinum concentrations, and standard curves were prepared using an identical procedure. Values reported are the average of three to five separate platinum determinations for each sample.

The platinum levels in all samples were estimated by atomic

absorption spectroscopy by flameless atomisation at 2700°C using a Perkin Elmer Spectrophotometer Model 2380 equipped with a HGA Graphite Furnace, automatic sampler and HGA 300 programmer. A three-stage temperature program with 50 s drying, 35 s charring and 7 s atomisation was used. Argon was the inert purge gas, and pyrolytically-coated graphite tubes were used in the furnace. Plasma samples were diluted 4:1 (v/v) with 0.1 mol/l HCl and injected straight into the furnace without any further treatment.

Statistical analysis

All experiments were repeated at least three times and representative results are presented. The significance of the difference between means was determined by analysis of variance [27]. Statistics were obtained via Students' *t*-test using group analysis when two comparisons were made.

RESULTS

Encapsulation efficiency of different liposomal preparations

Liposomal encapsulation of CDDP is limited by low water solubility (1 mg/ml) at room temperature. Increasing the temperature of the solution increases CDDP solubility, but this also increases the hydrolysis of the chloride groups of CDDP. On the positive side, the chloride group hydrolysis can be reduced to a minimum at high chloride concentrations (0.9% NaCl). On the negative side, it enhances the precipitation of cisplatin at room temperature, decreasing the solubility.

To optimise entrapment of the drug, we have attempted to encapsulate CDDP into various lipid compositions, hydrated with different hydration solutions (Table 1). In group I at 1 mg/ml CDDP in 0.9% saline, total encapsulation was 80 μg/ml lipid in phosphatidylcholine:phosphatidylserine:cholesterol (PC:PS:Chol). Lowering the saline concentration to 0.25% in the hydration solution increased the solubility of CDDP to 2 mg/ml, which increased the final encapsulation to 120 μg/ml lipid. The addition of 5% mannitol to the 0.25% saline hydration solution did not increase the solubility of CDDP (2mg/ml), but did increase the encapsulation to 210 μg/ml lipid. In group 2, increasing the lipid concentration slightly increased the encapsulation to 250μg/ml.

In group 3, the lipid and platinum concentration in hydration buffer were both increased and encapsulation was performed at 37°C constant temperature for 30 min as described in the Materials and Methods. This procedure yielded the highest cisplatin encapsulation (600 μg/ml lipid). All the studies reported here were performed with liposome preparation which contained 600 μg/ml of encapsulated CDDP and a lipid ratio of 3:3.1:3.7 of PC, PS and Chol. These liposomes were stable for at least 1 week at 4°C.

Table 1. *Effect of different lipid composition on encapsulation efficiency of cisplatin*

Liposomes					
Lipids composition	Molar ratio	Total lipid concentration (mg/ml)	Hydration solution (mg/ml)		Total encapsulation (μg/ml)
PC:PS:Chol	3:1:3	11	Saline (0.9%)	1	80
			Saline (0.25%)	2	120
			SM	2	210
PC:PS:Chol	3.4:1:3.8	22	SM	2	250
PC:PS:Chol	3:3.1:3.7	66	SM	3	600

PC = L-α-phosphatidylcholine; PS = L-α-phosphatidyleserine; Chol = cholesterol; SM = saline (0.25%) mannitol (5/0%).

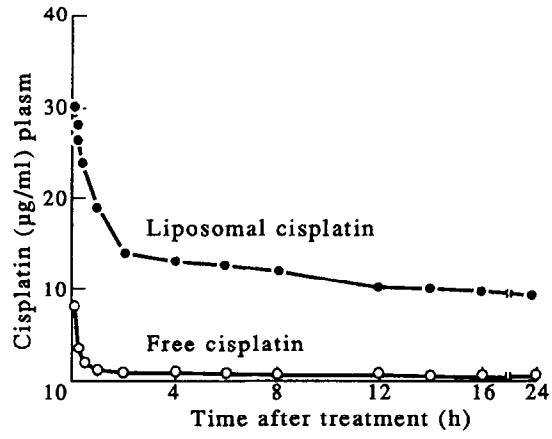

Fig. 1. Plasma pharmacokinetics of free cisplatin (○) or liposomal cisplatin (●) (6 mg/kg of body weight each). Levels of cisplatin in plasma were evaluated by atomic absorption spectroscopy. Values are means for three determinations.

In vitro *cytotoxic evaluations*

The cytotoxicity of the CDDP and L-CDDP preparations were evaluated *in vitro* using the L1210 mouse leukaemia and human ovarian (NIH, OVCAR) cell lines. With L1210 cells the IC_{50} of both CDDP and L-CDDP was 0.15 μg/ml at 48 and 72 h. The highest dose of 0.6 μg/ml resulted in more than 90% cytotoxicity of the cells when treated with either CDDP or L-CDDP preparations. The IC_{50} for NIH OVCAR was 0.05 μg/ml for both 24 and 72 h treatment with CDDP and L-CDDP. The cell cytotoxicity was similar at all doses used in these experiments either with free CDDP or L-CDDP (data not shown).

Plasma pharmacokinetics studies

The plasma pharmacokinetics of platinum levels was followed after the i.v. administration of CDDP or L-CDDP at a dose of 6 mg/kg body weight [6] using male CD_2F_1 mice (Fig. 1). The plasma platinum profiles of both CDDP and L-CDDP show a biphasic curve indicating a two-compartment fit of the plasma values. The CDDP treatment resulted in peak plasma concentration of 8 μg/ml at 5 min, while plasma levels of platinum with L-CDDP treatment were 30 μg/ml at 5 min showing a 4-fold increase of drug in plasma. The $t_{\frac{1}{2}}\alpha$ of 2 h and $t_{\frac{1}{2}}\beta$ of >48 h were observed with L-CDDP in contrast to $t_{\frac{1}{2}}\alpha$ of 15 min and $t_{\frac{1}{2}}\beta$ of 12 h with free CDDP treatment. At 24 h the plasma concentration of platinum with L-CDDP was 10 μg/ml, while no platinum with free CDDP was detectable after 10 h.

Tissue distribution of cisplatin

After a single i.v. dose (6 mg/kg body weight) of L-CDDP, the liver and spleen showed the highest peak concentration of the drug (4 h, 12 h, respectively) (Table 2). However, peak concentration of the CDDP level occurred at 5 min both in the liver and in the spleen. With L-CDDP the concentration of drug continued to increase in liver and spleen with time, eventually reaching levels 4 to 8-fold higher than after free CDDP. The drug levels in lungs and heart were similarly higher following treatment with L-CDDP than with CDDP, but not in kidney.

Table 2. Organ concentrations of cisplatinum after i.v. injection of CDDP or L-CDDP into mice*

Time	Liver		Spleen		Kidney		Lung		Heart	
	F	L	F	L	F	L	F	L	F	L
5	7.8	6.4	26.0	16.0	16.5	10.5	2.0	11.0	1.2	8.6
15	7.0	7.3	17.0	25.0	10.0	9.7	2.0	10.8	0.4	8.5
30	6.0	10.0	11.0	34.0	9.3	12.1	2.2	10.0	0.8	6.0
1 h	5.0	16.0	8.5	46.0	7.8	10.0	1.9	7.5	0.6	4.8
2 h	5.0	15.0	8.0	57.0	7.4	7.1	2.2	6.2	0.8	3.9
4 h	4.0	20.0	7.6	62.0	5.5	6.4	1.2	6.0	0.3	3.1
12 h	ND	12.0	ND	66.0	ND	5.4	ND	4.7	3.2	1.9
24 h	3.8	11.0	6.0	52.0	4.7	6.4	0.2	4.4	0.2	1.2
48 h	3.3	9.4	5.0	43.0	3.6	2.0	0.2	4.3	0.2	0.8

Values are means of four mice (μg/g tissue).
F = free cisplatin, L = liposomal cisplatin, ND = not done.
* Dose = 6 mg/kg body weight.

In vivo *antitumour evaluations*

An *in vivo* comparison of the route of administration and schedule dependency of varying doses of CDDP and L-CDDP in the treatment of murine ascitic L1210 leukaemia was performed (Table 3). L1210 cells, 1×10^5 were injected intraperitoneally into mice. A dose of 6 mg/kg i.v. in an intermittent schedule of 1, 5 and 9 days resulted in a treated vs. control (T/C) of 175% with CDDP, compared to a T/C of 113% with L-CDDP (experiment 1). However, the same schedule produced a T/C of 243% in each case with no long-term survivors when the doses of both CDDP and L-CDDP were raised to 9 mg/kg i.v. This indicates a comparable antitumour activity with CDDP and L-CDDP treatments (experiment 2). Using the same schedule, but varying the dose of drug produced no significant differences in antitumour activity with either CDDP or L-CDDP treatment (experiment 3). However, when a combination of i.p. and i.v. drug administration was utilised (experiment 4), an enhanced antitumour activity was obtained (T/C = 250% for both CDDP and L-CDDP) with three out of eight long-term survivors in each case. In addition, with the i.p. administration of high doses of drug on days 1 and 9, the L-CDDP provided a much higher therapeutic response with a T/C of 337% and three out of eight long-term survivors (Table 3). CDDP produced only 168% T/C with no long-term survivors. All the mice in the CDDP group showed drug-related toxicity, which was manifested in weight loss and general lethargy. All the deaths were apparently toxicity related.

Table 4. Nephrotoxicity evaluation of rats treated with free and liposomal-encapsulated cisplatin

Treatment	Dose (mg/kg)	BUN (mg/dl)	Creatinine (mg/dl)
Control	—	13.5 ± 0.93	0.48 ± .02
Free CDDP	3	85.4 ± 20.3	1.04 ± .17
L-CDDP	3	16.3 ± 1.2	0.48 ± .02
L-CDDP	4.5	11.3 ± 1.5	0.46 ± .02

Rats were treated with free cisplatin at a dose of 3 mg/kg on day 1 and 5 i.p. and with liposomal cisplatin at doses of 3 and 4.5 mg/kg on the same schedule. At day 9, all the rats were killed and BUN and creatinine levels were determined as described in Materials and Methods.
Values are expressed as mean ± S.E.

Nephrotoxic evaluation of CDDP and L-CDDP

Rats were treated i.p. with CDDP at 3 mg/kg dose on days 1 and 3; and L-CDDP was given at doses of 3 and 4.5 mg/kg on the same schedule.

Animals treated with free CDDP exhibited weight loss and general lethargy, whereas no such observation was made with animals treated with L-CDDP even at 1.5-times higher dose than free drug. The BUN levels at day 9 with free CDDP averaged 85.4 mg/dl compared to the values of 16.3 and 11.3 mg/dl with doses of 3 and 4.5 mg/kg of L-CDDP, respectively. The elevation of BUN concentration in rats treated with free drug was significantly higher compared to the control animals or those treated with liposomal drug ($P < 0.01$). The creatinine level in rats treated with free cisplatin demonstrated the same pattern as observed with BUN (Table 4).

The weight of kidneys in rats treated with free cisplatin was significantly less than control rats ($P < 0.01$). However, no such difference was noted in rats treated with L-CDDP either at 3 mg/kg or 4.5 mg/kg dose (Figs 2, 3). At day 9 after initiating the drug regimens, the ability of slices to accumulate [^{3}H]PAH was decreased significantly in the kidneys obtained from rats

Table 3. In vivo antitumour evaluation of CDDP and L-CDDP against L1210 ascites leukaemia in CD2F1 mice

Experiment no.	Treatment schedule			Dose (mg/kg)			Median survival (days)		T/C (%)		Long-term survivors*	
	Day 1	Day 5	Day 9	Day 1	Day 5	Day 9	CDDP (Range in parentheses)	L-CDDP (Range in parentheses)	CDDP	L-CDDP	CDDP	L-CDDP
1	IV	IV	IV	6	6	6	14(12–16)	9(8–9)	175	113	0(8)†	0(8)
2	IV	IV	IV	9	9	9	17(11–17)	17(12–17)	243	243	0(8)	0(8)
3	IV	IV	IV	12	3	3	15(10–15)	13(9–13)	187	162	0(8)	0(8)
4	IP	IV	IV	12	3	3	20(14–20)	20(15–20)	250	250	3(8)	3(8)
5	IP	—	IP	12	—	12	13.5(9–15.5)	27(18–27)	168	337	0(8)	3(8)

Male CD2F1 mice were injected i.p. with 1×10^5 L1210 leukaemia cells and drug treatment was given according to the schedule. Survival of tumour-bearing control mice was 7 days in experiment no. 2 and 8 days in all other experiments.
CDDP = free cisplatin; L-CDDP = liposomal cisplatin. * Long-term survival = 30 days. † Alive (no. treated).
IV = intravenous, tail vein.
IP = intraperitoneal.

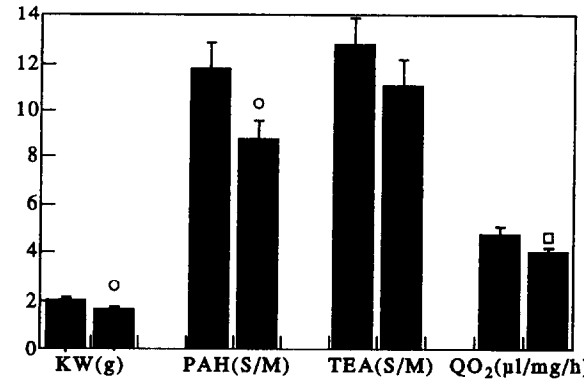

Fig. 2. The comparison of kidney weights (kw), PAH and TEA uptake and QO_2 consumption in the control rats and rats treated with free cisplatin (free CP) at a dose of 3 mg/kg. On the figure insert ○ indicates $P < 0.01$ and □ indicates $P < 0.05$.

receiving CDDP compared to the control ($P < 0.01$) (Fig. 2). In contrast, no significant changes in [^{3}H]PAH uptake occurred at any dose of L-CDDP (Fig. 3). The ability to accumulate [^{14}C]-TEA decreased consistently in rats treated with free CDDP although the differences were not statistically significant. There was no difference in [^{14}C]TEA accumulation in rats with L-CDDP doses when compared to the controls. The ability of slices to consume oxygen (QO_2) in the presence of glutamine decreased significantly in rats receiving free CDDP ($P < 0.05$). In contrast, no abnormality in oxygen consumption was noted in kidney slices of rats treated with L-CDDP.

DISCUSSION

CDDP is an anti-neoplastic platinum agent commonly used alone or in combination with other agents in a variety of chemotherapeutic regimens. In ovarian cancer, CDDP has shown extensive activity. Although initial response rates are as high as 98% with cisplatin-containing regimens, most patients are not completely cured of disease [28]. Also on the negative side, clinical application of CDDP can be severely limited by its dose-limiting toxic side-effects [3,29,30]. For both reasons, improvements in this form of treatment are necessary.

Some therapeutic regimens have been improved when the agent considered has been encapsulated in liposomes. While liposomal encapsulation of other chemotherapeutic agents has been shown to reduce toxicities associated with the administration of free drug [14–16, 31–33, 35], to date, liposomal encapsulation of CDDP has not attracted much attention [19]. The encapsulation of CDDP has produced some problems due to its lower water solubility and low encapsulation into liposomes [35]. However, our preparation resulted in a relatively high encapsulation due to a different lipid composition, higher total lipid concentration, and high concentration of CDDP in the hydration solution used in our preparations.

The *in vitro* cytotoxicity evaluation of our preparation of L-CDDP possessed similar cytotoxicity as CDDP against both murine (L1210) and human ovarian (NIH-OVCAR) carcinoma cell lines. The CDDP and L-CDDP IC_{50} values with NIH-OVCAR (0.05 µg/ml), however, were lower than their IC_{50} for L1210 (0.15 µg/ml). These relatively low IC_{50} values explain the cytotoxic similarities of the two preparations on each cell line, and the different IC_{50} values between the two cell lines may result from differences in tissue culture methodology.

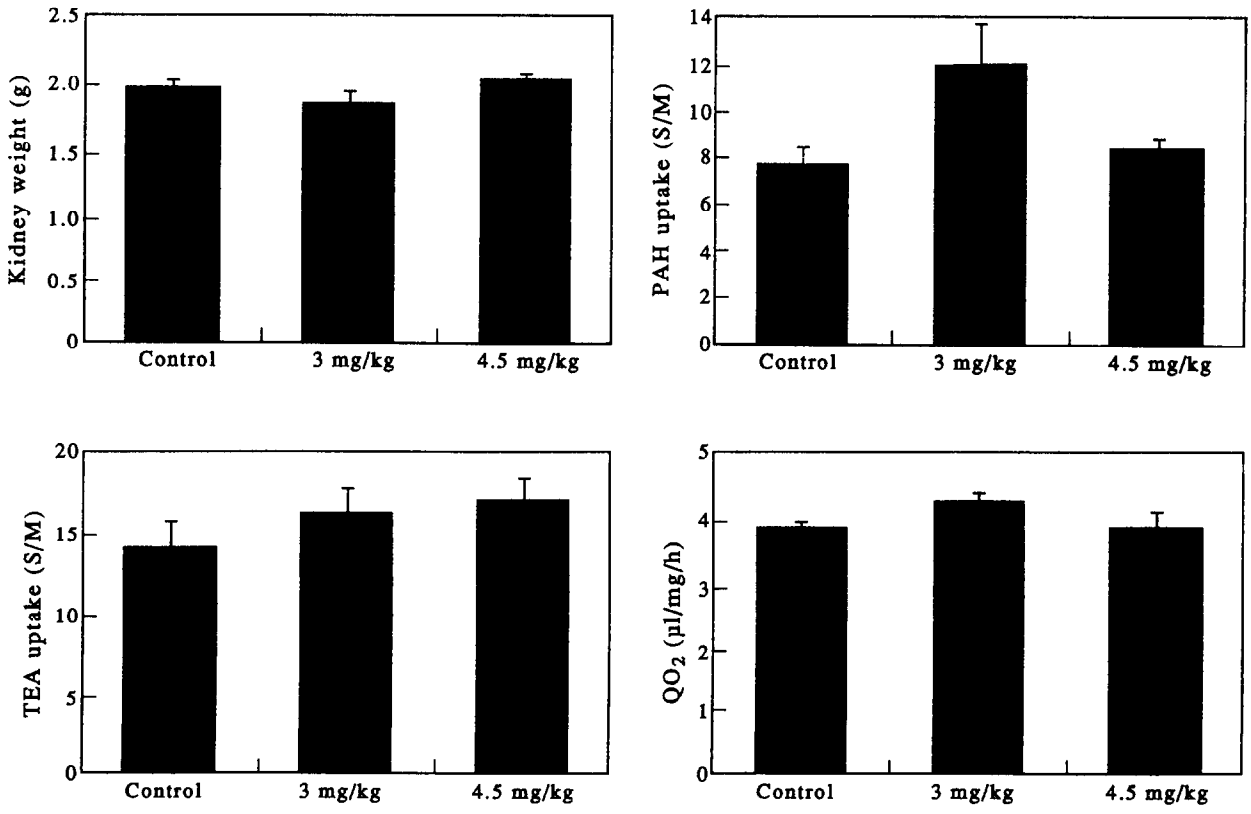

Fig. 3. The comparison of kidney weights, PAH and TEA uptake and QO_2 consumption in the control rats and rats treated with cisplatin encapsulated in liposomes at a dose of 3 and 4.5 mg/kg. There is no significant difference in any of the parameters between the controls and either group of treatment. See Materials and Methods for more details.

To determine the optimal dose and the most effective route of administration, we varied the dose range, time schedule and route of administration of the two preparations. Because of the nature of the tumour outgrowth in the peritoneum in our model, i.v. and i.p. administrations were investigated alone and in combination. With the i.p. route of administration, the efficacy of L-CDDP was increased 2-fold over that of CDDP. The differences between our *in vitro* cytotoxicity and the *in vivo* antitumour effects of equivalent doses of CDDP and L-CDDP may be due to the difference in drug release from liposomes and bioavailability to the cells.

In many ways, our results concerning the improved therapeutic benefits of L-CDDP simulate previous ones examining solid tumours [19]. The *in vivo* pharmacological studies indicate that the plasma concentration of both the CDDP and L-CDDP declined in a biphasic manner. However, the *in vivo* antitumour studies indicate that higher plasma levels of L-CDDP may not be all completely bioavailable, thereby providing less tumour response than free CDDP at lower doses. In the pharmacokinetic studies, we measured the total platinum content after i.v. administration. With i.v. administration, therapeutically effective platinum levels may not have been available to the tumour cells in the peritoneal cavity in *in vivo* experimentation.

We found the organ distribution of CDDP agrees with other studies examining CDDP and its analogues [34–39]; but in the present study, the platinum levels in the spleen and liver following L-CDDP treatments were several times higher than the levels achieved with CDDP. The explanation may lie in the greater uptake of L-CDDP by the reticuloendothelial system. This observation mimics other studies using other cytotoxic drugs encapsulated in liposomes [12,13,15]. This offers a greater potential for toxicity to these organs, however, this was not examined in the present study. Previously, we did not discern hepatotoxicity in rats treated with our preparation of L-CDDP [26]. On the other hand, the higher levels of L-CDDP could be useful in the hepatic maladies treated with CDDP.

In vivo injections of CDDP at doses of 3 mg/kg i.p. caused a higher elevation of BUN and creatinine, whereas no alterations were observed with L-CDDP i.p., even at a dose of 4.5 mg/kg. One could hypothesise that elevated BUN was due to an augmented urea production caused by CDDP rather than through a nephrotoxic event. However, our data favour the latter. Alterations in renal morphology have been observed in previous studies with CDDP [26], and the *in vitro* transport and metabolic functions in the present study are also consistent with nephrotoxicity of CDDP.

For the evaluation of nephrotoxicity, the ability of kidney slices to accumulate [^{3}H]PAH has been used as an indicator of tubular function [20, 22]. Therefore, it is not surprising that CDDP causes a significant decrease in PAH uptake by renal slices in this study. It is interesting to note that similar changes were not appparent in L-CDDP-treated rats even at a higher dose of 4.5 mg/kg. Metabolic functions are generally less sensitive to renal insult than transport functions, yet QO_2 was decreased substantially with CDDP and not with L-CDDP.

In conclusion, these studies indicate that liposomal encapsulation of CDDP can be useful in maintaining the therapeutic efficacy while reducing the nephrotoxicity associated with the administration of the free drug. This may have further clinical applications.

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Randomised Study of Immunotherapy with OK-432 in Uterine Cervical Carcinoma

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OK-432, a streptococcal preparation, was administered to patients with stage Ib and II cervical carcinoma except for adeno- and adenosquamous carcinomas. To evaluate the efficacy of OK-432 precisely, 177 patients were stratified by clinical stage, radiotherapy, and lymph node metastasis after complete radical hysterectomy and pelvic lymphadenectomy. Within each stratum, patients were divided randomly into OK-432 and control groups. 85 patients received OK-432 and 92 patients did not. No significant difference was observed in overall 5-year disease free rates between the OK-432 and the control groups, although the mean diameter of erythema on SU-polysaccharide (SU-PS) skin test was larger in the OK-432 group than in the control group. In stage IIb, a significant difference was observed between the OK-432 and control groups. This difference, however, could be attributed in part to the different incidence of the lymph node metastasis. In stage II without lymph node metastasis, 5-year disease free rate was significantly higher in the OK-432 group.
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INTRODUCTION

CARCINOMA OF the uterine cervix is the most common cancer among gynaecological malignant tumours in Japan. The decrease in mortality from cervical cancer during the past 25 years has been attributed to the detection of early stage cancer rather than to improvement in the treatment of invasive cancer, since screening for uterine carcinoma has become widespread. Five-year survival rate is 99% for carcinoma *in situ*, 87% for stage I and 68% for stage II in Japan [1]. Invasive cervical carcinoma is usually treated with radical hysterectomy and bilateral pelvic lymphadenectomy, and adjuvant postoperative radiotherapy is often employed in an effort to improve both local control and survival rate in high risk patients. However, even with optimal surgical results and/or radiation therapy, recurrent carcinoma may develop. Although improvement in local control of pelvic disease in patients with bulky tumours by using combined modality therapy has been reported, there has been no remarkable improvement in overall survival rate because of a corresponding increase in the occurrence of distant metastases [2, 3].

Generally the potency of cellular immunity is decreased in patients with carcinoma. Radiotherapy and chemotherapy give rise to further decreases in immune responsiveness. Numerous