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# Liposomes as drug carrier system for *cis*-diamminedichloroplatinum (II)

## II. Antitumor activity in vivo, induction of drug resistance, nephrotoxicity and Pt distribution

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**Summary.** In this study we have investigated the use of liposomes as a drug carrier system for *cis*-diamminedichloroplatinum(II) (cis-DDP) in order to reduce the nephrotoxicity with preservation of antitumor activity. Liposomes (PC/PS/Chol 10:1:4) were prepared using hydration media containing no or a relatively low concentration of NaCl. It was found that cis-DDP containing liposomes (lip cis-DDP) injected i.v. to IgM immunocytoma-bearing LOU/M rats at a dose of 1 mg cis-DDP/kg (cumulative dose 7 mg cis-DDP/kg) showed less antitumor activity than the free drug. The optimal cumulative dose of free cis-DDP for induction of antitumor activity in this tumor system is 7 mg/kg (7 × 1 mg/kg). At a dose of 2 mg lip cis-DDP/kg (cumulative dose 14 mg cis-DDP/kg) the antitumor activity was almost equal to that of free cis-DDP. The antitumor activity could not be increased by choosing another phospholipid composition of the liposomes [DPPC/DPPG/Chol (10:1:10)]. cis-DDP incorporated in DPPC/DPPG/Chol liposomes showed a similar antitumor activity to cis-DDP incorporated in PC/PS/Chol liposomes. After an i.v. dose of 2 mg lip cis-DDP/kg (PC/PS/Chol) kidney damage was less compared to the treatment with free cis-DDP (1 mg/kg). However, after a single dose of 2 mg cis-DDP/kg or a cumulative dose of 8 or 16 mg cis-DDP/kg, kidneys of rats treated with lip cis-DDP contained twice as much Pt as after treatment with free cis-DDP. Moreover, after treatment with lip cis-DDP, a twofold increase of the amount of Pt in tumor tissue was measured. In vitro studies with Pt recovered from spleens obtained from rats treated with lip cis-DDP i.v. showed that based on the equal amounts of Pt recovered the antitumor activity of the recovered Pt was reduced, indicating inactivation of cis-DDP in vivo. As during treatment with free cis-DDP, recurrence of the tumor was observed during the continued treatment with lip cis-DDP. It was found that these recurrent tumors were resistant to further therapy with cis-DDP. In conclusion, cis-DDP encapsulation into liposomes decreased the nephrotoxicity. The antitumor activity of cis-DDP is preserved by liposome encapsulation when it was used at a dose of 2 mg/kg, but it was reduced in terms of earlier onset of regrowth.

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*Abbreviations used:* cis-DDP, *cis*-Diamminedichloroplatinum(II); Pt, platinum; PC, egg L-α-phosphatidylcholine; PS, bovine L-α-phosphatidylserine; DPPC, L-α-dipalmitoylphosphatidylcholine; DPPG, L-α-dipalmitoylphosphatidylglycerol; Chol, cholesterol; lip *cis*-DDP, *cis*-DDP encapsulated in liposomes; PL, phospholipid; AAS, atomic absorption spectroscopy. RES, reticuloendothelial system

### Introduction

With the object of enhancing the therapeutic value of antineoplastic drugs, liposome incorporation of drugs has been studied for several years (reviewed in [27, 41]). Promising results have been reported for the encapsulation of doxorubicin [7, 8, 10, 11, 16, 26, 28, 29], arabinofuranosylcytosine [12, 21, 24, 30, 33] and methotrexate [19, 20, 22, 31]. It is suggested that encapsulation of methotrexate and arabinofuranosylcytosine induces antitumor activity against drug-resistant tumors, as reported for drug-resistant subcell lines of the murine lymphoma TLX5 and the P1798 mouse lymphosarcoma both in vitro and in vivo [20, 22, 30]. But in the L1210 tumor model, entrapped arabinofuranosylcytosine did not overcome drug resistance [34]. As well-defined and stable liposomes are becoming available [2], the clinical application of liposome-encapsulated drugs seems possible in the near future.

Cis-DDP has demonstrated a remarkable chemotherapeutic potential in a variety of tumor models in laboratory animals [35] and in human neoplasms, such as testicular and ovarian cancer and cancer of the lung and head and neck [23, 35]. However, a restriction on the use of cis-DDP is its renal toxicity [13, 14, 23]. Therefore, studies were performed with the objective of developing new platinum analogues [32]. In addition, it has already been shown that treatment schedules using prehydration and forced diuresis limit the cis-DDP nephrotoxicity [5].

A relatively small number of attempts have been made to study the effects of encapsulation of cis-DDP into liposomes [9, 18, 25, 40, 43]. Satisfactory encapsulation of cis-DDP is thwarted by its low water solubility and its low lipophilicity. We showed that cis-DDP-containing liposomes PC/PS/Chol 10:1:4 prepared by hydration with 0.9% NaCl were very leaky [37]. Furthermore, they contained relatively low amounts of encapsulated cis-DDP. Decreasing the NaCl content of the hydration medium made it possible to produce liposomes that were very stable with respect to drug leakage (for more than 50 days at 4° C) and showed relatively high binding capacities (encapsulation efficiency of 9.8% or 18%, using a hydration medium of 0.2% NaCl/4.2% mannitol or 5% mannitol, re-

spectively). However, exclusion of NaCl from the solvent for cis-DDP resulted in decomposition of the drug because of displacement of the chloride ligands by water molecules, leading to the formation of cationic aquation products [15]. This decomposition of cis-DDP, which may reduce the antitumor activity of the drug, can be prevented by adding a minimal amount of 0.1% NaCl in the solvent for cis-DDP [15]. We have shown that on a molar base, encapsulated cis-DDP, compared with the free drug, is less cytotoxic against a murine gastric aquamous cell carcinoma in vitro [37]. However, after leakage or forced liberation from the liposomal structure, cis-DDP and its cationic aquation products were found to be as active as free cis-DDP. In this study we investigated the antitumor activity, nephrotoxicity and organ distribution of platinum, of liposome-encapsulated cis-DDP in IgM immunocytoma-bearing LOU/M rats. As in this tumor model cis-DDP induces drug resistance [17], we wished to find whether liposome encapsulation of cis-DDP could prevent the induction of Pt resistance. For liposomal encapsulation cis-DDP was dissolved in 0.2% NaCl/4.2% mannitol or 5% mannitol, resulting in the liposomal Pt presence as cis-DDP or cationic Pt aquation products [15], respectively.

## Materials and methods

*Animals.* Breeding pairs of LOU/M Wsl rats and the transplantable IgM immunocytoma of LOU/C Wsl origin were kindly provided by Dr. H. Bazin (Catholic University, Louvain, Belgium) [1]. Animals were bred under specified pathogen-free conditions at the National Institute of Public Health and Environmental Hygiene, Bilthoven, the Netherlands. Female rats were used at an age of 10–16 weeks when they weighed 160–190 g. Animals were maintained according to accredited conditions in our facility and enjoyed uniformly good health at the initiation of the studies.

*Tumor model.* LOU/M Wsl rats were inoculated s.c. on the left flank with $1 \times 10^4$ IgM immunocytoma cells in 0.5 ml plain RPMI 1640 medium (Grand Island Biological Co., Europe B. V., Hoofddorp, The Netherlands). Details of the tumor model are described elsewhere [17]. Briefly animals inoculated with $1 \times 10^4$ cells develop a palpable tumor after 14–17 days, which grows within the next 6–7 days to a diameter of 25–35 mm. At 20–25 days after inoculation, the tumor has metastasized to the regional lymph nodes, and micrometastases in the liver can be detected. The growth of the tumor was measured twice a week with vernier callipers and recorded as the mean of three perpendicular measurements.

*Drugs. cis*-Diamminedichloroplatinum (II) (cis-DDP) was kindly provided by Dr H. Meinema (Institute of Applied Chemistry, TNO, Utrecht, The Netherlands) and by Dr D. de Vos (Pharmachemie B. V., Haarlem, The Netherlands). For experiments with free or liposome-encapsulated cis-DDP, the drug was dissolved in 5% mannitol or in 0.2% NaCl/4.2% mannitol.

*Preparation of liposomes.* Multilamellar vesicles (MLV) were formed by using the classic "film" method [3]. The phospholipids (PL) used were obtained from Sigma Chemical Co. (St Louis, Mo): egg L-α-phosphatidylcholine-type V-E (PC), bovine brain L-α-phosphatidylserine (PS), dipalmitoyl-L-α-phosphatidylcholine (DPPC), dipalmitoyl-L-α-phosphatidylglycerol (DPPG), cholesterol (Chol). The compositions of the phospholipid bilayers were (on a molecular basis): PC/PS/Chol 10:1:4 and DPPC/DPPG/Chol 10:1:10. Details are described elsewhere [37]. Free (non-liposome-encapsulated) cis-DDP was removed by application of a recently developed method using the cation exchange resin Dowex 50W-X4 (analytical grade, 200–240 mesh, converted to the sodium form, (Serva, Heidelberg, FRG) for 5 min (minimum 4 g Dowex/ml dispersion) [38]. The resin was separated from the liposome-containing supernatant by filtration through 8.0-μm membrane filters (Uni-pore, Biorad, Richmond, Calif). After the Dowex procedure, the mean diameter and the polydispersity index of the extruded liposomes were determined by dynamic light scattering (Nanosizer, Coulter Electronics Ltd., Luton, UK). The polydispersity was expressed as an index on a scale from 0 to 9. Zero indicates to a monodisperse and 9 to an extremely polydispersed dispersion [3]. The polydispersity index of the liposome dispersions used in this study ranged from 2 to 4 [37]. The liposome dispersions were stored at 4–6° C under nitrogen and kept protected from the light.

The phospholipid concentration of liposome suspensions was determined by measuring phosphate according to the method of Fiske and Subbarow [6]. It was found that the preparation procedure was accompanied by a phospholipid loss of 20% ± 7.5% ($n = 23$). The mean phospholipid concentration of the liposome dispersion (PC/PS/Chol) was 35.0 ± 3.3 μmol/ml ($n = 23$). The zeta-potential of liposomes was determined by microelectrophoresis (Rank Brothers, Mark II, Bottisham, UK).

*Pt determination in liposomes.* The concentration of Pt in cis-DDP liposomes was determined by atomic absorption spectroscopy (AAS) (Perkin Elmer 400, Norwalk, Conn) at 295 nm (split size 0.7 nm) with an air/acetylene flame. Details are described elsewhere [37].

*Determination of Pt in plasma and tissue.* Pt was determined in body fluids (plasma and cell suspensions) and tissues by atomic absorption spectroscopy with electrothermal atomization in a graphite furnace (ET-AAS) using a Model 451 Video AAS (Instrumentation Laboratory Inc., Wilmington, Mass). Tissue samples were digested by an enzymatic digestion procedure (De Groot and Wubs, submitted for publication). Both tissue digests and plasma samples were analyzed after dilution of the sample into an appropriate range and modification of the final matrix [4]. Detection limits of the respective procedures were 25 μg Pt/kg for tissues and 5 μg Pt/l for plasma.

*Distribution study.* IgM immunocytoma-bearing rats received i.v. injections of 2 mg/kg of either cis-DDP or lip cis-DDP. After 4, 24, 48 and 120 h animals ($n = 6$) were autopsied. Blood samples were collected from the abdominal aorta and plasma, spleen, kidney, liver and tumor were isolated, and organs were weighed. About 0.5 g of intact tissue was used for determination of the Pt concentration by AAS.

*Histopathology.* Kidneys were fixed in 4% (w/v) formaldehyde in 0.067 *M* Sörensen buffer, pH 6.9. After embedding in glycol methacrylate 1-μm sections were prepared and

stained with Giemsa and periodic acid silver methenamine (PASM). The slides were read under code to avoid reader bias.

*In vitro assessment of antitumor activity of Pt recovered from spleen.* Four hours after i.v. injection of lip cis-DDP (2 mg/kg) 3 rats were killed, their spleens were removed aseptically, were weighed and a sample of each spleen was taken for measuring the Pt content by AAS. Next, spleens were pooled and spleen cells were isolated by gently pressing organ fragments through a 60-μm mesh nylon screen adding 10 ml plain medium E [42]. Pt was recovered from cells and liposomes by three cycles of freezing (−20° C) and thawing (RT°). For separation of cellular and liposomal debris from the released Pt the suspension was centrifuged by 200000 *g* and the supernatant isolated. From this supernatant the Pt concentration and antitumor activity was determined. Free cis-DDP (5 μg/ml suspension) was added to part of a control spleen suspension from untreated rats, whereas the remaining part was used to prepare a control supernatant containing no cis-DDP. Both spleen cell suspensions were processed as described as above.

The antitumor activity of the various supernatants was measured in vitro in a murine gastric squamous cell carcinoma 5DO4. Briefly, $1 \times 10^4$ tumor cells were cultured in polystyrene 96-well tissue culture culsters with flat-bottom wells (cat. no. 3596, Costar, Cambridge, Mass, USA). After 24 h the wells were washed twice with medium E supplemented with 10% heat-inactivated fetal calf serum (FCS; Flow Laboratories, UK), 0.002 *M* glutamine, streptomycin (100 μg/ml), penicillin (100 IU/ml), and fungizone (0.25 μg/ml). Supernatant of spleens from lip cis-DDP-treated animals and supernatant of spleens from control animals with or without addition of free cis-DDP (see above) was added at various concentrations as indicated in Fig. 8. In addition, free cis-DDP diluted in supernatant was also added at various concentrations to the 5DO4 cells. All supernatants were removed after 24 h incubation at 37° C. Therafter the tumor cells were washed and refreshed with supplemented medium E and the tumor cell monolayers were labeled with 5 μCi methyl-$^3$H-thymidine ($^3$H-TdR, specific activity 5 Ci/mmol; Radiochemical Centre, Amersham, Bucks., UK) per ml culture for 4 h. For removal of unbound $^3$H-TdR tumor cell monolayers were washed with phosphate-buffered saline (pH 7.2) for 30 s using a multiple cell culture harvester (Skatron, Lierbyen, Norway). After washing, the tumor cells were lysed in 0.1 ml sodium dodecyl sulfate (SDS) solution (0.2%). The lysates were transferred into scintillation vials containing 1 ml of a mixture of Insta Fluor (Packard-Becker B. V., Groningen, The Netherlands) and Triton X-100 (5:3) and measured in an ISO cap/300 liquid scintillation counter (Nuclear, Chicago Corp., Des Plaines, Ill). Determinations were performed in triplicate wells. The antitumor activity was expressed as percentage growth inhibition (GI): $GI = (1 - T/C) \times 100\%$, where T is counts per minute (cpm; median of the triple wells) of $^3$H-TdR incorporated after incubation of tumor cell monolayers under test conditions (Pt-containing supernatant obtained from spleen from lip cis-DDP-treated animals, supernatant from control spleen cells to which cis-DDP was added or supernatant to which cis-DDP was diluted); C cpm of $^3$H-TdR incorporated after incubation of tumor cell monolayers under control conditions (supernatant of spleen cells from nontreated rats).

## Results

*Antitumor activity in vivo of liposome-encapsulated cis-DDP*

*Liposomes (PC/PS/Chol) prepared with 5% mannitol.* Rats with tumors about 25 mm in diameter ($n=6$) were treated with 1 or 2 mg free cis-DDP per kg body weight (in 5% mannitol) by i.v. injection or with the same dose of cis-DDP encapsulated in liposomes (lip cis-DDP) twice a week for 3 weeks (cumulative doses of 7 or 14 mg cis-DDP/kg, respectively). Both doses of free cis-DDP induced complete tumor regression starting after the second injection (Fig. 1). No difference in antitumor activity was observed between 1 and 2 mg cis-DDP/kg (cumulative doses of 6 and 12 mg/kg, respectively). Rats treated with 2 mg/kg cis-DDP died after six injections. Because they died almost free of tumor, this was probably caused by the toxicity of the drug, as indicated by the decrease in body weight (Fig. 2). However, at a dose of 1 mg lip cis-DDP/kg only tumor growth retardation was observed and eventually animals died of the tumor disease. At a dose of 2 mg lip cis-DDP/kg tumor regression was similar to the regression induced by the free drug during the first 14 days. After day 14 recurrence of the tumor was observed, and at day 21, three out of six animals died because of extensive tumor growth and metastases. As shown in Fig. 2, loss of body weight was only observed during treatment with the free drug.

*Liposomes (PC/PS/Chol) prepared with 0.2% NaCl/4.2% mannitol.* Lip cis-DDP prepared by hydration with 5% mannitol appeared to induce less antitumor activity than the free drug (Fig. 1). This might be due to partial inactivation as a result of decomposition of cis-DDP because of the displacement of the chloride ligands by water molecules leading to the formation of cationic aquation products [15]. A comparison was made between the antitumor activity in vivo of cis-DDP liposomes prepared by hydra-

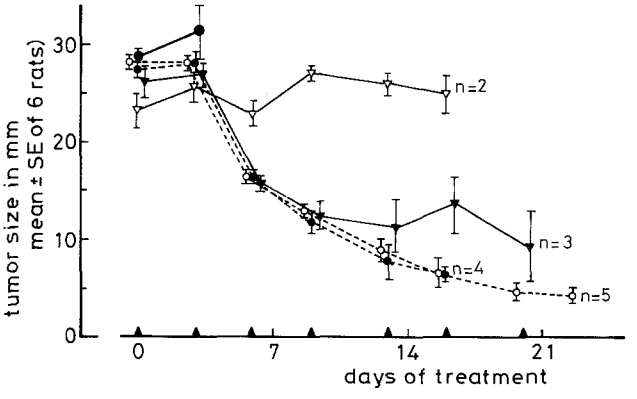


**Fig. 1.** Antitumor activity of cis-DDP and lip cis-DDP (PC/PS/Chol 10:1:4) (5% mannitol) in solid IgM immunocytoma bearing LOU/M rats. Tumor cells ($1 \times 10^4$) were inoculated sc. Tumor growth during therapy is presented (mean ± SE of 6 animals, but indicated by n if less animals were present). The drug was administered i.v. twice weekly (▲) ●——● no treatment; ○-----○ 1 mg cis-DDP/kg; ●-----● 2 mg cis-DDP/kg; ▽——▽ 1 mg lip cis-DDP/kg; ▼——▼ 2 mg lip *cis*-DDP/kg

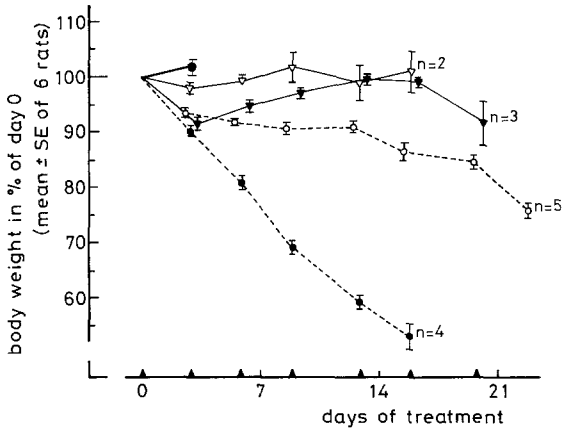


**Fig. 2.** Effect of cis-DDP and lip cis-DDP (PC/PS/Chol 10:1:4) (5% mannitol) on the body weight of solid IgM immunocytoma bearing rats. Results are expressed as the mean percentage increase or decrease in body weight (mean ± SE of 6 rats) with respect to the body weight on day 0 (100%). Data on the effect on body weight were derived from the experiment represented in Fig. 1. ●——● no treatment; ○-----○ 1 mg cis-DDP/kg; ●-----● 2 mg cis-DDP/kg; ▽——▽ 1 mg cis-DDP/kg; ▼——▼ 2 mg lip cis-DDP/kg

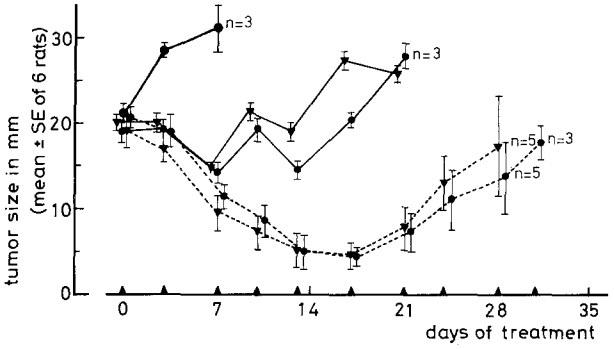


**Fig. 3.** Effect of the presence of 0.2% NaCl in the hydration medium on the antitumor activity of lip cis-DDP in solid IgM immunocytoma bearing LOU/M rats. Tumor cells ($1 \times 10^4$) were inoculated sc. Tumor growth during treatment is presented (mean ± SE of 6 animals, but indicated by n if less animals were present). Lip cis-DDP liposomes (PC/PS/Chol 10:1:4) were administered i.v. twice weekly (1 mg cis-DDP/kg) (▲). ●——● No treatment; ▼-----▼ cis-DDP in 0.2% NaCl/4.2% mannitol; ●-----● cis-DDP in 5% mannitol; ▼——▼ lip cis-DDP in 0.2% NaCl/4.2% manntiol; ●——● lip cis-DDP in 5% mannitol

tion with 5% mannitol and cis-DDP liposomes prepared by hydration with 0.2% NaCl/4.2% mannitol. The treatment (1 mg lip cis-DDP/kg) was started when the tumor had reached a mean diameter of 20 mm. No difference was observed between the antitumor activity of free cis-DDP dissolved in 5% mannitol or in 0.2% NaCl/4.2% mannitol (Fig. 3). Tumor regression was observed after the second injection and was almost complete at day 14. However, at day 21 resistance to cis-DDP therapy resulted in regrowth of the tumor. After an initial regression, lip cis-DDP-treated tumors showed regrowth even as soon as day 7. No differences were found between both liposome preparations. In separate experiments with lip cis-DDP prepared by hydration with 0.2% NaCl/4.2% mannitol also a dose of 2 mg lip cis-DDP/kg was tested (Fig. 4). The free cis-DDP had similar antitumor activity to that recorded earlier. At a dose of 2 mg lip cis-DDP/kg, the antitumor activity was almost the same as that observed for the free drug. Complete regression was observed within 14 days, but regrowth of the tumor started almost immediately after that time period. For rats treated with 2 mg free cis-DDP/kg regrowth of the tumor showed a delay of approximately 7 days compared with lip cis-DDP-treated tumors prepared by hydration with 0.2% NaCl/4.2% mannitol.

*Liposomes (DPPC/DPPG/Chol) prepared with 0.2% NaCl/4.2% mannitol.* As can be concluded from the results presented above, decomposition of cis-DDP was probably not responsible for the reduced antitumor activity of lip cis-DDP; replacement of 5% mannitol by 0.2% NaCl/4.2% manntiol (which prevented hydrolysis of cis-DDP) did not improve the antitumor activity of lip cis-DDP. Therefore, we investigated whether the reduced antitumor activity of encapsulated cis-DDP might be a result of the phospholipid composition. Therefore, the antitumor activity of DPPC/DPPG/Chol (10:1:10) liposomes [36] was studied in comparison with the antitumor activity of both the free drug and the drug encapsulated in the fluid type liposomes (PC/PS/Chol) (Fig. 5). The treatment of immunocytoma-bearing rats with 1 mg cis-DDP liposomes/kg body weight resulted in an antitumor activity that was less than the activity of the free drug (Fig. 5). Although the initial antitumor activity of cis-DDP in liposomes at a dose of 2 mg/kg body weight was similar to that of the free drug, regrowth of the tumor started at day 17. No difference in antitumor activity was observed between the solid and the fluid-type liposomes at both dose regimens.

*Cis-DDP resistance*

As indicated by the regrowth during continued therapy, resistance to cis-DDP therapy was induced by free cis-DDP as well as by cis-DDP encapsulated in liposomes (Figs. 3–5). In order to prove that the tumor cells developed resistance during lip cis-DDP treatment (2 mg/kg) (Fig. 5), tumor cells were isolated at day 28 and frozen in liquid nitrogen for further studies. Cells collected from

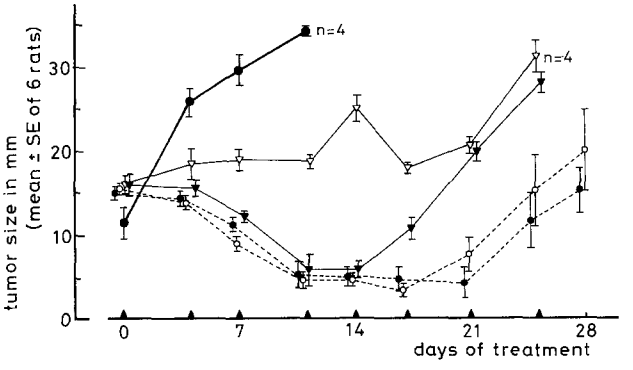


**Fig. 4.** Antitumor activity of cis-DDP and lip cis-DDP (PC/PS/Chol 10:1:4) in solid IgM immunocytoma-bearing LOU/M rats. Tumor cells ($1 \times 10^4$) were inoculated s.c. Tumor growth during treatment is presented (mean ± SE of 6 animals unless otherwise indicated). cis-DDP was dissolved in 0.2% NaCl/4.2% mannitol. Drug was administered i.v. twice weekly, 1 mg and 2 mg cis-DDP/kg (▲). ●——●, No treatment; ○-----○, 1 mg cis-DDP/kg; ●-----●, 2 mg cis-DDP/kg; ▽——▽, 1 mg lip cis-DDP/kg; ▼——▼, 2 mg lip cis-DDP/kg

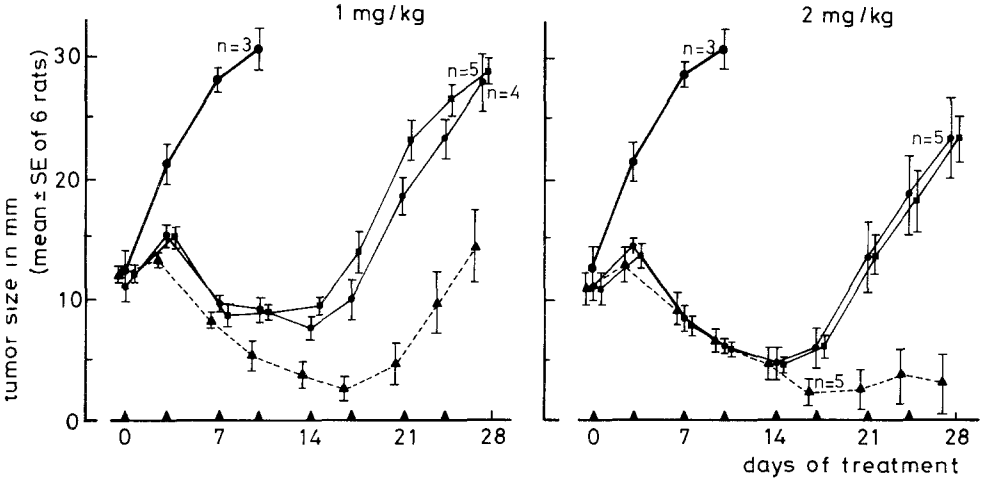


**Fig. 5.** Antitumor activity of lip cis-DDP (DPPC/DPPG/Chol 10:1:10) compared with lip cis-DDP (PC/PS/Chol 10:1:4) and free cis-DDP in solid IgM immunocytoma-bearing LOU/M rats. Tumor growth during treatment is presented (mean ± SE of 6 animals unless otherwise indicated). Drug was administered i.v. twice weekly (1 mg and 2 mg cis-DDP/kg) (▲). cis-DDP was dissolved in 0.2% NaCl/-4.2% mannitol. ●——●, No treatment; ▲-----▲, cis-DDP; ■——■, lip cis-DDP (PC/PS/Chol); ●——●, lip cis-DDP (DPPC/DPPG/Chol)

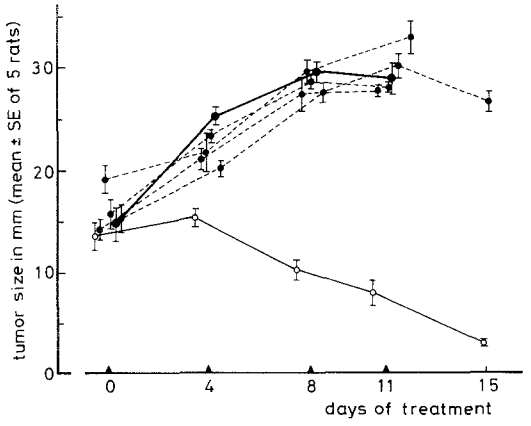


**Fig. 6.** Induction of cis-DDP resistance during treatment with lip cis-DDP (PC/PS/Chol) in solid IgM immunocytoma-bearing LOU/M rats. IgM immunocytoma cells isolated from four tumors showing regrowth during lip cis-DDP treatment (see text and Fig. 4), were inoculated ($2.5 \times 10^4$) in normal LOU/M rats. At a tumor size of about 15 mm, animals were treated i.p. with cis-DDP (1 mg/kg) (▲). Tumor growth during treatment is presented (mean ± SE of 6 animals). ○——○, Parent IgM immunocytoma line; ●——●, cis-DDP-resistant IgM immunocytoma subline; ●-----●, IgM immunocytoma cells isolated from 4 different tumors after regrowth during lip cis-DDP treatment

four tumors were s.c. inoculated in the flank of four groups of LOU/M rats ($n = 5$). After development of a tumor (±15 mm diameter), free cis-DDP (1 mg/kg) was injected i.p. twice a week for 2 weeks. Figure 6 shows that cis-DDP injected animals with the parent cell line was very effective, while cis-DDP injected animals with a cis-DDP resistant subline did not induce tumor regression. Cis-DDP treatment of animals inoculated with tumor cells isolated from growing tumors during lip cis-DDP treatment, did not result in regression of these tumors. So, as with free cis-DDP [17], the treatment of IgM immunocytoma-bearing rats with lip cis-DDP resulted in the induction or selection of cis-DDP-resistant tumor cells.

*Nephrotoxicity of free cis-DDP and lip cis-DDP*

Histopathological examination was performed on kidneys from the animals treated in the experiment depicted in Fig. 4, in which cis-DDP was administered in free form or encapsulated in liposomes. The animals were autopsied after 4 weeks, the cumulative dose being 8 or 16 mg cis-DDP/kg. The Pt concentrations in the kidneys after treatment with doses of 1 and 2 mg free cis-DDP/kg were 34.3±5.5 and 71.7±6.7 mg/kg dry weight, respectively, and those after treatment with doses of 1 and 2 mg lip cis-DDP/kg were 72.1±6.0 and 127.2±4 mg/kg dry weight, respectively. As judged from the Giemsa-stained sections, a high incidence of focal alterations of the epithelium of the proximal tubules was found in the kidneys of the animals receiving doses of 2 mg free cis-DDP/kg (Table 1). The epithelial cells of these tubules contained many polymorphic degenerative nuclei or bizarre hypertrophic nuclei. The appearance of the cytoplasm was indicative of hydropic degeneration. Observation of the PASM-stained

**Table 1.** Semiquantitative histopathological evaluation of kidney lesions[a] in LOU/M rats after repeated i.v. administration of cis-DDP or cis-DDP entrapped in liposomes (PS/PC/Chol)

| Focal alterations of proximal tubules[b] | Control | cis-DDP | | Lip cis-DDP | |
|---|---|---|---|---|---|
| | 0 | 1[c] | 2 | 1 | 2 |
| None | 5/5[d] | | | 3/4 | 4/6 |
| Mild | | 6/6 | | 1/4 | |
| Moderate | | | | | 2/6 |
| Marked | | | 5/5 | | |

[a] Slides were scored at random under coded numbers
[b] For description of lesions see text
[c] Dose (mg/kg) administered in each injection. Cumulative doses at autopsy were 8 mg/kg and 16 mg/kg for the 1 mg/kg and 2 mg/kg dose groups, respectively
[d] Number of animals with lesion versus total number of animals investigated

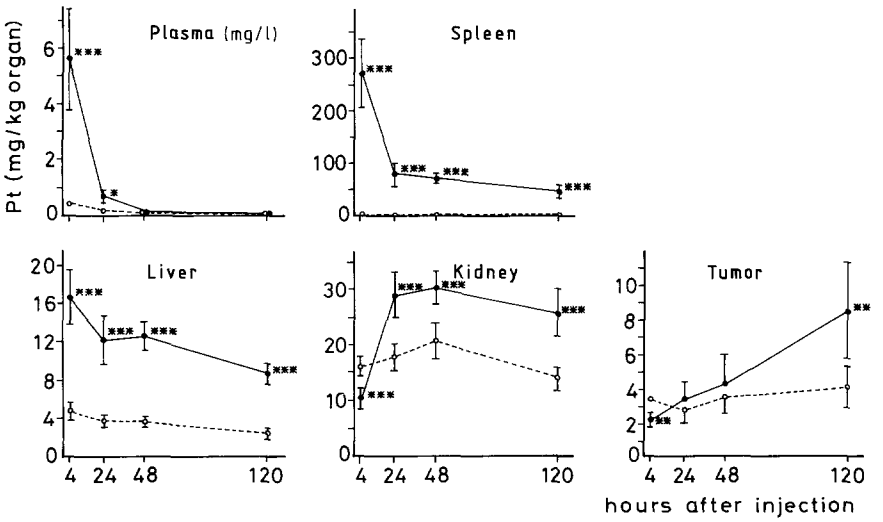


**Fig. 7.** Concentration of Pt in plasma and tissue after administration of cis-DDP or lip cis-DDP in solid IgM immunocytoma-bearing LOU/M rats. Tumor cells ($1 \times 10^4$) were inoculated s.c. At a mean tumor diameter of 17 mm rats (6 per group) were injected i.v. with 2 mg cis-DDP or lip cis-DDP (PC/PS/Chol)/kg. Rats were autopsied at 4, 24, 48 and 120 h after administration of the drug. Pt concentration was expressed in mg Pt/kg dry weight tissue or mg Pt/l plasma. ●——●, lip cis-DDP (0.2% NaCl/4.2% mannitol); ○- - - -○, cis-DDP in 0.2% NaCl/4.2% mannitol. Lip cis-DDP vs cis-DDP: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$

**Table 2.** Pt content of different organs of LOU/M rats (percentage of injected Pt) after a single i.v. injection of 2 mg/kg cis-DDP or lip cis-DDP

| Organ | 4[a] cis-DDP/ lip cis-DDP | 24 cis-DDP/ lip cis-DDP | 48 cis-DDP/ lip cis-DDP | 120 cis-DDP/ lip cis-DDP |
|---|---|---|---|---|
| Spleen | 0.15[b]/17.2 | 0.15/ 5.4 | 0.21/ 5.5 | 0.23/ 3.9 |
| Liver | 4.4 /16.9 | 3.4 /13.0 | 3.3 /13.2 | 2.4 / 8.6 |
| Kidney | 2.7 / 2.0 | 2.8 / 4.6 | 3.2 / 4.6 | 2.1 / 3.8 |
| Tumor | 1.8 / 0.93 | 1.4 / 2.0 | 1.5 / 2.3 | 0.66/ 1.8 |
| Total | 9.1 /37.0 | 7.8 /25.0 | 8.2 /25.6 | 5.4 /18.1 |

[a] Time (h) after a single i.v. injection

[b] SD values (6 animals per group) are not shown for reasons of clarity, but can be derived from Fig. 7

sections, in particular, allowed the conclusion that the basal lamina of these tubules were obviously thickened, probably due to tubular collapse. In most cases, the alterations of the tubules were accompanied by a slight infiltration of mononuclear cells. Compared with the 2 mg free cis-DDP/kg group, the incidence and severity of the lesions in the 1 mg free cis-DDP/kg or 1 and 2 mg lip cis-DDP/kg groups were much less pronounced (Table 1). In the control group no lesions were observed.

*Pt concentration in plasma and target organs*

After a single i.v. injection of 2 mg free cis-DDP/kg body weight the drug disappeared from plasma within between 4 and 48 h (Fig. 7; Table 2). After i.v. injection of 2 mg lip cis-DDP/kg body weight the Pt concentration in plasma, 4 h after injection was about 13 times higher than after injection of free cis-DDP. The drug disappeared rapidly in between 4 and 48 h after the injection. After 120 h, in both groups the Pt concentration in plasma had reached the reference level, which was found in non-treated rats to be $0.02 \pm 0.01$ mg Pt/l (mean ± SD).

After injection of either free cis-DDP or lip cis-DDP the highest Pt concentrations in the liver were observed at 4 h. However, the Pt concentration was about 4 times higher after administration of lip cis-DDP than after administration of free cis-DDP.

In the spleen, after free cis-DDP administration a rather flat Pt course was found, which reached a maximum concentration after 48 h ($3.2 \pm 0.8$ mg Pt/kg dry weight). A completely different course was observed when cis-DDP was administered encapsulated in liposomes. The highest Pt concentration after lip cis-DDP was observed already after 4 h ($272 \pm 68$ mg Pt/kg dry weight). The concentration at that time was 86 times higher than the maximum concentration found 48 h after free cis-DDP injection. After 24 h the Pt concentration in spleen had decreased to about one-third of the value present at 4 h. A similar difference in distribution of Pt was found between free cis-DDP and lip cis-DDP when injected to non-tumor-bearing rats (data not shown).

In kidney, both after free cis-DDP and after lip cis-DDP administration maximum Pt concentrations were reached after 48 h. After free cis-DDP administration a high affinity of the drug and/or its degradation products for this organ was observed, which resulted in a Pt concentration in kidney that was 5–7 times higher than in the other organs during the period of 4–48 h after the injections. After lip cis-DDP administration the initial uptake of Pt was lower than after the administration of free drug. However, from 24 h onward the Pt concentration in kidney was twice as high after lip cis-DDP than after cis-DDP administration.

In tumors a constant Pt concentration was obtained throughout the experimental period of 4–120 h after free cis-DDP administration. As in the kidney, the initial uptake of cis-DDP in the tumor after lip cis-DDP administration was lower than after injection of the free drug. However, in contrast with the free cis-DDP group, the Pt con-

centration in the tumor increased steadily during the observation period. If the concentration of Pt in the tumor was expressed as Pt per total organ, concentration-time curves similar in shape were obtained (data not shown). Therefore, it was concluded that the time courses of the Pt concentration in tumor were hardly affected by the increasing tumor size due to growth of the tumor.

*Antitumor activity of Pt isolated from spleen*

Besides the lipid composition, another reason for the reduced antitumor activity of lip cis-DDP might be inactivation of cis-DDP by cells of the reticuloendothelial system (RES) in liver and spleen, as most of the injected lip cis-DDP was present in these organs (Table 2). Therefore, 4 h after i.v. injection (2 mg lip cis-DDP/kg), Pt was recovered from spleen. The spleen cell population was the first choice, as a high accumulation of Pt (17% of the injected dose of cis-DDP) was found present in the spleen at 4 h after i.v. injection of lip cis-DDP (2 mg/kg; Table 2). However, animals treated with free cis-DDP could not be used as controls, because of the very low levels of Pt in the spleen (see Fig. 7). Therefore, as a control, free cis-DDP was added to control spleen cells and to control spleen cell supernatants. Supernatants derived from spleen homogenates after three cycles of freezing and thawing and a centrifugation step at 200000 *g* contained about 63%±5% (mean ± SD) of the original Pt content of the spleen. They were incubated in vitro with the 5DO4 tumor cell line to test their antitumor activity. These experiments were repeatedly carried out ($n$= 5). Results of a representative experiment are shown in Fig. 8. In terms of Pt-concentration, a four-fold increase of Pt was needed in spleens recovered from lip cis-DDP treated rats compared with the cis-DDP added to spleen cells or spleen cell supernatants obtained from spleens of nontreated control rats for a similar level of cytotoxicity to be attained.

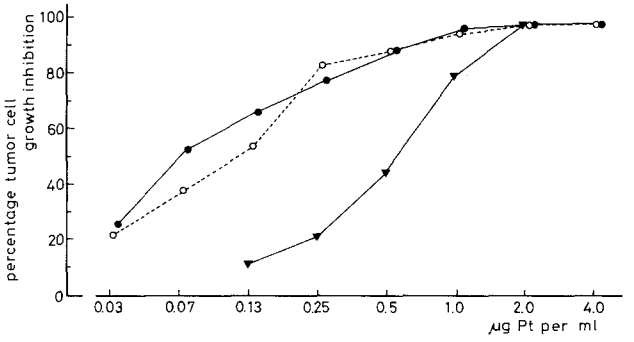


**Fig. 8.** Effect of Pt recovered from the spleen of lip cis-DDP (PC/PS/Chol 10:1:4) treated LOU/M rats on tumor cell growth in vitro. Antitumor activity was measured against a monolayer culture of a murine gastric squamous cell carcinoma (5DO4). Inhibition of the tumor cell growth was measured by postlabeling with $^3$H-TdR. As a control on the binding of recovered cis-DDP to cell fragments, 5 µg cis-DDP/ml was added to spleen cell suspension obtained from spleens of control rats. As supernatant of control spleen cells appeared to have some inhibitory effect on the growth of the tumor cells, tumor cell monolayers used as controls were cultured in supernatants derived from control spleens. In addition, cis-DDP was diluted in supernatant derived from control spleens. ●———●, cis-DDP added to control supernatant; ○- - - - -○, cis-DDP added to spleen cell suspension and processed (3 cycles of freezing and thawing and centrifugation) with spleen cells; ▼———▼, cis-DDP recovered (after 3 cycles of freezing and thawing and centrifugation) from spleen 4 h after i.v. injection of 2 mg lip cis-DDP/kg

## Discussion

So far, only a small number of studies have been reported concerning the encapsulation of cis-DDP in liposomes. These studies have had different goals and have yielded conflicting data about the antitumor activity. In this study we used well-characterized liposomes [37] to investigate the applicability of cis-DDP encapsulation in liposomes for the reduction of the nephrotoxicity with no reduction of the antitumor activity. We have shown [37] that liposomes (PC/PS/Chol 10:1:4) prepared by hydration with 0.9% NaCl internalize low amounts of cis-DDP and leak the encapsulated drug rapidly within 7–14 days. When the NaCl concentration of the hydration medium was reduced it was possible to prepare lyposomes that were highly stable against drug leakage and contained a relatively high concentration of cis-DDP. Liposome-encapsulated cis-DDP showed less cytotoxicity in vitro, but on the basis of cis-DDP leakage, in vitro cytotoxicity against a murine gastric squamous cell carcinoma was equivalent. Results of the present study show that encapsulation of cis-DDP into liposomes decreased the in vivo antitumor activity of cis-DDP compared with the activity of free cis-DDP (Figs. 1, 3–5). It was observed that only the concentration of 2 mg lip cis-DDP/kg administered i.v. induced almost similar antitumor activity to 1 and 2 mg/kg free cis-DDP mg/kg (Figs. 1, 4, 5). However, the antitumor activity observed with 1 and 2 mg free cis-DDP/kg can be measured even after a dose of 0.5 mg/kg [17]. The reduced antitumor activity of lip cis-DDP was independent of the hydration medium used or of the phospholipid composition (Figs. 3–5). Similar results were obtained by Muzya et al. [25], who had to double the dose of lip cis-DDP (20 mg/kg) in the i.v. treatment of the Crocker's sarcoma in mice. Better results were found when lip cis-DDP administration was combined with local hyperthermia [43]. For this purpose lipids with specific lipid transition temperature were used for the cis-DDP liposome preparation. The uptake of cis-DDP by the tumor and tumor growth inhibition of sarcoma 180 was increased after treatment of the mice with a combination of lip cis-DDP and local hyperthermia treatment [43]. Outstanding results were reported for the treatment of the Ehrlich ascites carcinoma in the mice by i.p. administration of cis-DDP encapsulated in neutrally charged liposomes [40]. The percentage life-span increased to 140% for mice treated with lip cis-DDP, in contrast to the 24% increase in the case of treatment with the free drug. It was suggested that these results were due to the entry of liposomes into the tumor cells. This suggestion was in agreement with in vitro experiments, which showed that after treatment with free cis-DDP 85% of the Ehrlich carcinoma cells were viable, whereas the viability of the lip cis-DDP-treated cells was only 18% [40]. This observation indicates that the antitumor activity in vitro against Ehrlich carcinoma cells may be due to more than just cis-DDP leakage from the liposomes, a process that was responsible for the in vitro antitumor activity against 5DO4 carcinoma cells shown in our previous study [37].

The relative differences in pharmacokinetic profile after injection of lip cis-DDP and free cis-DDP are compa-

rable with the results described by Freise et al. [9]. Our results show that cis-DDP liposomes are rapidly cleared from the circulation especially by liver and spleen, giving rise to a Pt concentration in the liver of 4 times and in the spleen 115 times that found after administration of the free drug. Also, administration of cis-DDP encapsulated in liposomes resulted in a higher Pt content in kidney and tumor than after treatment with free cis-DDP. According to Freise et al. [9], this increase of Pt content is probably due to the still active circulatory cis-DDP released from the circulating liposomes. In our experiments liver and spleen together contained at 4 h after lip cis-DDP administration 34% of the injected dose. Liposomes are cleared from the circulation mainly by RES cells of spleen and liver. On the basis of information obtained in experiments with DXR-containing liposomes, we suggest that after endocytosis by macrophages of the spleen or by Kupffer cells of the liver, liposomes will be degraded [36, 39], which may result in a sustained release of cis-DDP or related species from the cells into the blood. Such a sustained release might explain the steady increase in Pt content in kidney and tumor depicted in Fig. 7. However, our data on the antitumor activity indicate that the high concentrations of Pt found in plasma and tumor are of no relevance for the degree of antitumor activity. The same apparent discrepancy can also be observed for the Pt concentration and induced toxicity in the kidney. After repeated administration of cis-DDP or lip cis-DDP (cumulative dose 8 and 16 mg/kg) a higher concentration of Pt was found in the kidney after treatment with lip cis-DDP (Fig. 4). It was therefore very surprising to observe fewer kidney lesions than in the kidneys of animals treated with the free drug.

It might be speculated that after uptake by the RES from spleen and liver, cis-DDP is transformed into Pt species that might have less antitumor activity and induce less nephrotoxicity. To check this hypothesis, the activity of Pt present in the spleen after lip cis-DDP administration was further investigated. Following three cycles of freezing and thawing, we recovered approximately 63%±5% of the Pt stored in the spleen tissue. We tested this recovered Pt for its antitumor activity in vitro. It was remarkable to note that in repeated experiments, a reduced antitumor activity in vitro was measured. Combined with the fact that liposomal cis-DDP does not lose its antitumor activity by forced release from liposomes [37], our results indicate that the antitumor activity in vivo of cis-DDP after incorporation in liposomes was partly reduced by inactivation of the spleen by the RES. This is in contrast to the observations obtained for DXR by Storm et al. (submitted for publication), who demonstrated that liposomal DXR is not inactivated after phagocytosis by macrophages of the peritoneal cavity. The discrepancy in antitumor activity between DXR and cis-DDP after encapsulation might suggest that the efficiency of the drug in vivo depends on the way phagocytic cells process a drug. It seems that DXR is released by macrophages without losing its antitumor activity, while the antitumor activity of cis-DDP is reduced by phagocytic cells in the spleen.

Some studies [20, 22, 30] have suggested that the induced resistance to cytostatic agents might be prevented by incorporation of the drug into liposomes. Resistance against cis-DDP is easily induced in the IgM immunocytoma [17]. After treatment of tumor-bearing rats with free or lip cis-DDP, recurrences of the IgM immunocytomas occurred. When transplanted to naive recipient LOU/M rats, these immunocytoma sublines remained resistant to cis-DDP therapy. Therefore, with this tumor model no indications were found that encapsulation of cis-DDP into liposomes may overcome drug resistance.

In conclusion, the antitumor-activity of cis-DDP after encapsulation into liposomes may be partly inhibited by inactivation of cis-DDP, due to uptake by the RES, as we demonstrated for lip cis-DDP released from spleen cell populations. Although the mechanism is not clear, it might be proposed that inactivation involves dissociation of the highly reactive chloride ligands. To study this more in depth, it might be of interest to encapsulate in liposomes the cis-DDP derivative carboplatin (*cis*-diammine-1,1-cyclobutane dicarboxylate platinum II, CBDCA; JM8), in which the chloride ligands are already substituted by the cyclobutane carboxy group. The present pharmacokinetic data show that, most probably as a result of sustained release of Pt from spleen and liver, a two-fold increase of Pt in tumor tissue occurs after treatment with lip cis-DDP. Therefore, if the nephrotoxicity and antitumor activity are based on two different mechanisms, it is worth investigating how the antitumor activity of lip cis-DDP could be improved.

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