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Liposomes as a drug carrier system for *cis*-diamminedichloroplatinum(II). I. Binding capacity, stability and tumor cell growth inhibition in vitro

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Summary

We investigated the potential of liposomes as drug carrier for *cis*-diamminedichloroplatinum(II) (*cis*-DDP). The binding capacity, Pt release on storage, and in vitro antitumor activity was determined. It was found that the encapsulation efficiency was dependent on the NaCl concentration of the hydration medium in which *cis*-DDP was dissolved. In addition, *cis*-DDP liposomes prepared by hydration with 0.9% NaCl showed a relatively high drug leakage on storage. For *cis*-DDP liposomes prepared by hydration with 0.2% NaCl/4.2% mannitol or 5% mannitol the fraction of the drug associated with the liposomes was more than 90% after prolonged storage at 4°C for 50 days. Compared to the activity of free *cis*-DDP, *cis*-DDP encapsulated in liposomes showed a decreased antitumor activity in vitro against a murine gastric squamous cell carcinoma. This suggested that the antitumor activity of liposomal *cis*-DDP was due to leakage of *cis*-DDP from the liposomes. However, *cis*-DDP recovered from PC/PS/Chol liposomes after 3 cycles of freezing and thawing was equally active as free *cis*-DDP. From the present study it is concluded that *cis*-DDP liposomes prepared by hydration with 5% mannitol or 0.2% NaCl/4.2% mannitol have a relatively high binding capacity, and high stability against drug leakage.

Introduction

In general, studies with antineoplastic agents encapsulated in liposomes have shown that liposomal encapsulation of cytostatic drugs may result in preserved antitumor activity and reduced toxicity (Poste et al., 1984, Weinstein and Leserman, 1984). However, there is considerable doubt on the stability of drug-carrying liposomes as pharmaceutical product (Frøkjaer et al., 1982; Crommelin and Bommel, 1984; Bommel and Crommelin, 1984). Recent studies with doxorubicin

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(DXR)-containing liposomes emphasized the need for more insight into typical pharmaceutical aspects such as loading capacity, characterization in terms of lipid composition, charge and stability of DXR-containing liposomes (Crommelin et al., 1983; Crommelin and Van Bloois, 1983; Bommel and Crommelin, 1984; Hoesel et al., 1984; Jansen et al., 1985).

Another antineoplastic drug of interest for encapsulation is *cis*-diamminedichloroplatinum(II) (*cis*-DDP). Its usefulness, however, is mainly restricted by a severe dose-dependent nephro- and neurotoxicity (Loehrer and Einhorn, 1984; Finley et al., 1985). *cis*-DDP has very low hydrophilicity and lipophilicity. These characteristics of *cis*-DDP cause a number of problems associated with the encapsulation of this drug in liposomes especially with regard to the binding capacity and drug retention during storage (Freise et al., 1982). For *cis*-DDP-liposomes in general a very low binding capacity (Kaledin et al. 1981; Freise et al., 1982; Muzya et al., 1982; Yatvin et al., 1982) and a high drug leakage has been found (Freise et al., 1982).

In this study we attempted to increase both the binding capacity and stability against drug leakage of liposomes loaded with *cis*-DDP. In addition, to observe whether liposomal encapsulation influences the antitumor activity of the drug, *cis*-DDP encapsulated in liposomes and *cis*-DDP recovered from liposomes were tested for their antitumor activity in vitro against a murine gastric squamous cell carcinoma.

Materials and Methods

Drugs

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 NaCl solution (range 0.2–0.9% NaCl) which were made iso-osmotic with plasma by addition of mannitol.

Preparation of liposomes

Multilamellar vesicles (MLV) were formed by using the classical "film" method (Crommelin et al., 1983). The lipids used were obtained from Sigma Chemical Co. (St.Louis, MO): egg L-α-phosphatidylcholine type V-E (PC), bovine brain-L-α-phosphatidylserine (PS), L-α-dipalmitoylphosphatidylcholine (DPPC), L-α-dipalmitoylphosphatidylglycerol (DPPG), and cholesterol (Chol). The compositions of the phospholipid bilayers were (on a molar basis): PC/PS/Chol 10 : 1 : 4 and DPPC/DPPG/Chol 10 : 1 : 10. The phospholipids and cholesterol, dissolved in chloroform, were evaporated to dryness in a rotary evaporator under reduced pressure at 40–45 °C. The lipid-film was evacuated for at least 2 h. Then glass beads and the hydration medium containing 2.5 mg *cis*-DDP/ml were added. Different hydration media in which *cis*-DDP was dissolved at 50 °C were used: 5% mannitol, 0.2% NaCl/4.2% mannitol and 0.9% NaCl. The lipid film was hydrated by gently shaking at 50 °C and left after complete dispersion at room temperature under nitrogen for one night. At this stage of preparation the dispersion contained 44 μmol PL/ml. The liposomes were sized by extrusion through polycarbonate membrane filters with pore diameters of 0.6 and 0.2 μm (Uni-pore, Bio-Rad, Richmond, CA) using nitrogen pressures up to 0.8 MPa. By this extrusion procedure, *cis*-DDP precipitates were also removed. 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, F.R.G.) for 5 min (minimally 4 g Dowex/ml dispersion) (Storm et al., 1985). The resin was separated from the liposome containing supernatant by filtration through 8.0 μm membrane filters (Uni-pore, Bio-rad, Richmond, CA). 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, U.K.). The polydispersity index ranged from 0 to 9, zero indicates a monodisperse and 9 an extremely polydisperse dispersion (Crommelin et al., 1983). The liposome dispersions were stored at 4–6 °C under nitrogen and kept protected from light.

The phospholipid concentration of liposome suspensions was determined by measuring phosphate according to the method of Fiske and Subbarow (1925). It was found that the preparation procedure was accompanied by a phospholipid loss of $20.0 \pm 7.5\%$ ($n = 23$). The mean phospholipid concentration of the liposome dispersion (PC/PS/Chol) was 35.0 ± 3.3 μmol/ml ($n = 23$). The ζ-potential of liposomes was determined by microelectrophoresis (Rank Brothers, Mark II, Bottisham, U.K.) at 25°C. ζ-Potentials were calculated from averaged mobilities of at least 20 particles in both directions. Care was taken to focus on the stationary levels. As a rule, mobilities were determined at both the upper and the lower stationary level.

Pt determination

The concentration of Pt in *cis*-DDP liposomes was determined by atomic absorption spectroscopy (AAS) (Perkin Elmer 400, Norwalk, CN) at 295 nm (split size 0.7 nm) with an air/acetylene flame. The amount of liposome-encapsulated *cis*-DDP was determined by separating free *cis*-DDP from liposome-associated *cis*-DDP by gel filtration on Sephadex G-50 fine columns (30×1 cm Pharmacia, Uppsala, Sweden). Free *cis*-DDP was eluted at a volume of 18.5–28.0 ml, whereas the opalescent liposome suspension was eluted at a volume of 6.8–14.4 ml, so a complete separation of free and liposome associated *cis*-DDP was obtained. For AAS determination of the Pt content of liposomes 50 μl 20% Triton X-100 in water (BDH Chemicals Ltd., Poole, U.K.), 1000 μl 16.2 mg/ml lanthane nitrate ($La(NO_3)_3.9H_2O$, Merck Darmstadt, F.R.G.) was added to 200 μl liposome dispersion; the final volume was adjusted to 3000 μl with 1% HCl. Standards were prepared in 1% HCl containing Triton X-100, lanthane nitrate and empty liposomes in identical concentrations.

Assessment of antitumor activity in vitro

Antitumor activity was measured in vitro using a murine gastric squamous cell carcinoma line 5D04. This cell line was originally induced at our Institute in the stomach of a thymectomized and splenectomized BALB/c mouse after repeated oral administration of 3-methylcholanthrene. Tumor cells were adapted to tissue culture, stored at liquid nitrogen and used in their 8th to 20th in vitro passage (De Jong et al., 1984). For measuring tumor cell growth inhibition induced by *cis*-DDP, 1×10^4 tumor cells were cultured (37°C, 5% CO_2) in triplicate in 200 μl medium E (Williams and Gunn, 1974) supplemented with 10% heat inactivated fetal calf serum (FCS; Flow Laboratories, U.K.), 0.002 M glutamine, penicillin (100 IU/ml), streptomycin (100 μg/ml) and fungizone 0.25 μg/ml (further referred to as supplemented medium E).The cells were cultured in polystyrene 96-well tissue culture clusters with flat-bottomed wells (cat no. 3596, Costar, Cambridge, MA). After 24 h the wells were washed twice with supplemented Medium E, and *cis*-DDP or *cis*-DDP liposomes were added at various concentrations as indicated in the figures. *cis*-DDP was removed after 2 or 24 h incubation by gently washing the tumor cell monolayers twice with supplemented medium E. Thereafter the tissue culture medium was refreshed with supplemented medium E and the tumor cell monolayers were postlabeled with 5 μCi methyl-[^{3}H]thymidine ([^{3}H]TdR, spec. act. 5 Ci/mmol; Radiochemical Centre, Amersham, Buckinghamshire, U.K.) 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 of sodium dodecyl sulphate (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 Plaine, IL). Determinations were performed in triplicate. The antitumor activity was expressed as percentage growth inhibition (GI): $GI = (1 - T/C) \times 100\%$, where GI is percentage growth inhibition; T, cpm (median of triplicate results) of [^{3}H]thymidine incorporation after incubation of tumor cell monolayers under test conditions (addition of various dosis-forms of *cis*-DDP); C, cpm of [^{3}H]thymidine incorporation after incubation of tumor cell monolayers under control conditions (without addition of *cis*-DDP).

Results

Influence of sodium chloride content of the hydration medium on the binding capacity of cis-DDP to liposomes

Phospholipid films (PC/PS/Chol) were hydrated with *cis*-DDP solutions containing varying concentrations of NaCl (range 0.0–0.9%). Except for *cis*-DDP in 0.9% NaCl the hydration media were adjusted to an isotonic level with mannitol. Fig. 1 shows that hydration of the phospholipid film with 0.9% NaCl resulted in a binding capacity of 4.7 ± 0.8 μg *cis*-DDP/μmol phospholipid. By decreasing the percentage NaCl to 0.2% a slight but significant increase of the binding capacity was observed (up to 7.0 ± 0.9 μg *cis*-DDP/μmol phospholipid). When *cis*-DDP was dissolved in 5% mannitol without NaCl, 12.9 ± 0.8 μg *cis*-DDP/μmol phospholipid was encapsulated. The percentages of the amount of *cis*-DDP initially to the preparation that becomes liposome-associated (encapsulation efficiency) using 0.9% NaCl, 0.2% NaCl/4.2% mannitol or 5% mannitol were 6.4 ± 1.3, 9.8 ± 2 and 18.0 ± 1.6 respectively.

Hincal et al. (1979) have demonstrated that in a solution of *cis*-DDP in 5% mannitol water partially displaces the chloride ligands of *cis*-DDP leading to the formation of cationic mono- and diaquo species. In addition, they showed that the presence of at least 0.1% NaCl largely prevents this dissociation of *cis*-DDP. The question was raised whether such a dissociation of *cis*-DDP would reduce the antitumor activity of *cis*-DDP containing liposomes. Therefore, in our further studies liposomes were investigated with 3 types of hydration media differing in NaCl concentration: dispersions were prepared by hydration with 0.9% NaCl, with 0.2% NaCl/4.2% mannitol or with 5% mannitol. After sizing and free drug removal, these 3 liposome preparations were investigated with respect to their stability against drug leakage and particle size stability during long-term storage at 4–6 °C.

Long term storage of cis-DDP liposomes

Fig. 2 shows that the drug latency i.e. the fraction (%) of the drug actually associated with the liposomes was constant for *cis*-DDP liposomes prepared by hydration with 5% mannitol or 0.2% NaCl/4.2% mannitol during the 50 days of storage. Incidentally, liposomes prepared by hydration with 5% mannitol were measured at day 70

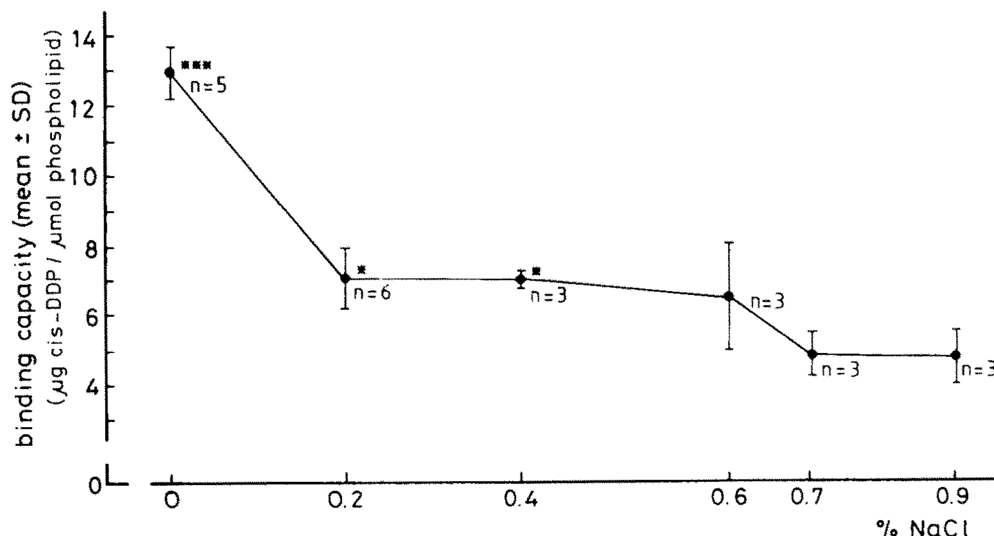

Fig. 1. Effect of NaCl content of the hydration medium on the binding capacity of *cis*-DDP to liposomes (PC/PS/Chol 10 : 1 : 4). % NaCl present in the hydration medium was 0.0–0.9. *n* is the number of individual experiments. Mean ± S.D. are shown. Except for 0.9% NaCl, all the hydration media were adjusted to physiological osmolarity with mannitol. * $P < 0.05$, *** $P < 0.001$. Differences with respect to 0.9% NaCl were analyzed by Student's *t*-test (two-sided).

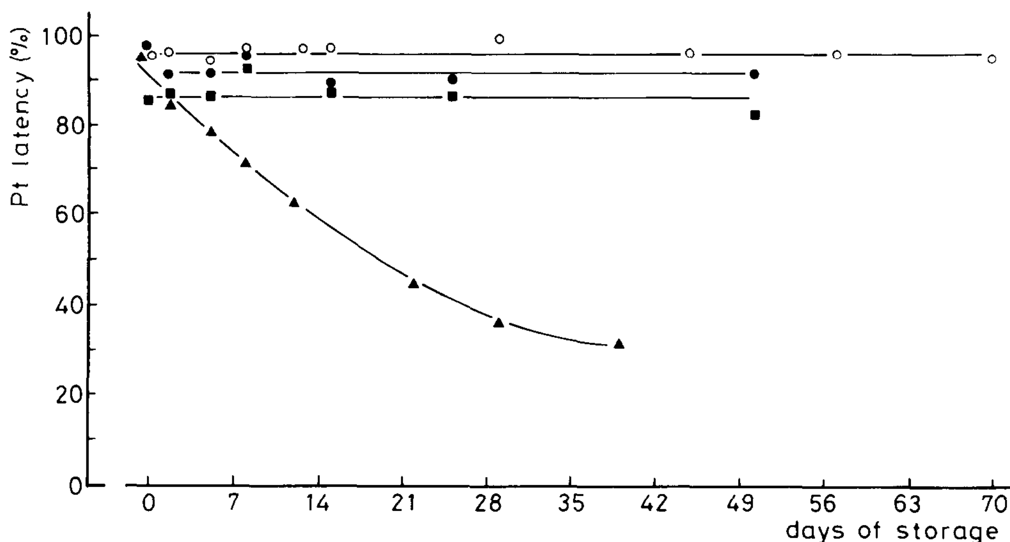

Fig. 2. Stability of *cis*-DDP containing liposomes against drug leakage during prolonged storage at 4° C. Pt-latency is expressed as percentage of the drug, present in the dispersion, which is liposome-associated. Mean of duplicate dispersions was determined. (●——●), PC/PS/Chol (10 : 1 : 4) in 5% mannitol; (■——■), PC/PS/Chol (10 : 1 : 4) in 0.2% NaCl/4.2% mannitol; (▲——▲), PC/PS/Chol (10 : 1 : 4) in 0.9% NaCl; (○——○), DPPC/DPPG/Chol (10 : 1 : 4) in 0.2% NaCl/4.2% mannitol.

and day 150; at these days no leakage was found (data not shown). However, liposomes prepared by hydration with 0.9% NaCl exhibited a high release of *cis*-DDP. At day 7, about 70% and at day 40 only about 30% of the initially incorporated amount *cis*-DDP was still liposome-associated.

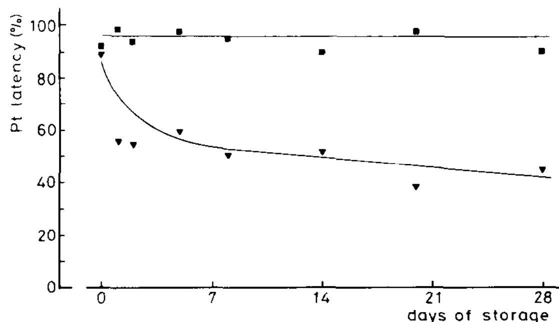

Fig. 3. Effect of the presence of mannitol on the stability of *cis*-DDP liposomes against drug leakage during prolonged storage at 4° C. The stability of *cis*-DDP liposomes was expressed as Pt latency (%). Mean of duplicate dispersions was determined. (■——■), PC/PS/Chol (10 : 1 : 4) in 0.2% NaCl. Concentration 233 μg *cis*-DDP/ml, binding 6.5 μg *cis*-DDP/μmol phospholipid, mean diameter 0.19 μm, polydispersity factor 3–4. (▼——▼), PC/PS/Chol (10 : 1 : 4) in 0.9% NaCl/4.2% mannitol. Concentration 166 μg/ml, binding 4.3 μg cis-DDP/μmol phospholipid, mean diameter 0.30 μm, polydispersity factor 3.

No important changes of the liposome diameter were observed during storage of the liposome preparations as determined by dynamic light scattering: the mean diameter found was at day 0: 0.28 μm, at day 70: 0.26 μm (5% mannitol), at day 0: 0.29 μm, and at day 50: 0.27 μm (0.2% NaCl/4.2% mannitol). No aggregation or fusion of liposomes could be detected as the polydispersity index was between 3 and 4 during the observation time of over 50 days.

In order to study whether the absence of mannitol was related to the high leakage of *cis*-DDP liposomes prepared by hydration with 0.9% NaCl, the leakage of liposomes prepared by hydration with 0.9% NaCl/4.2% mannitol was compared with that of liposomes prepared in 0.2% NaCl. Fig. 3 shows that addition of mannitol did not reduce the drug leakage of *cis*-DDP liposomes prepared in 0.9% NaCl. Moreover, no drug leakage was observed during 28 days when liposomes were prepared by hydration with only 0.2% NaCl. So, the *cis*-DDP leakage seems to be correlated to the NaCl concentration present in the hydration medium in which *cis*-DDP was dissolved.

TABLE 1

Characteristics of cis-DDP liposomes

Lipid composition Fluidity Hydration medium	PC/PS/Chol (10:1:4) fluid 5% mannitol	PC/PS/Chol (10:1:4) fluid 0.2% NaCl/ 4.2% mannitol	PC/PS/Chol (10:1:4) fluid 0.9% NaCl	DPPC/DPPG/Chol (10:1:10) solid 0.2% NaCl/ 4.2% mannitol
Concentration *cis*-DDP (μg/ml) [a]	451 ± 41 ($n = 5$)	246 ± 51 ($n = 6$)	159 ± 41 ($n = 3$)	398 ± 52 ($n = 7$)
Encapsulation efficiency (%) [a,b]	18.0 ± 1.6 ($n = 5$)	9.8 ± 2.0 ($n = 6$)	6.4 ± 1.6 ($n = 3$)	15.9 ± 2.1 ($n = 6$)
Binding capacity (μg *cis*-DDP/μmol PL) [a]	12.9 ± 0.8 ($n = 5$)	7.0 ± 0.9 ($n = 6$)	4.7 ± 0.8 ($n = 3$)	10.7 ± 1.2 ($n = 6$)
Size (μm)	0.25 ± 0.04 ($n = 3$)	0.29 ± 0.02 ($n = 6$)	0.30 ± 0.13 ($n = 3$)	0.34 ± 0.01 ($n = 8$)
Polydispersity index [c]	3–4	2–4	4–6	3
ζ-Potential (mV)	−28 (14) [e]	−19 (18) [e]	−14 [d] (6) [e]	−22 (7) [e]
pH	4–5	4–5	4–5	4–5

[a] Measured after extrusion procedure and Dowex treatment.
[b] Percentage *cis*-DDP encapsulated in the final product calculated from the start concentration (2.5 mg/ml).
[c] This index ranges from 0 to 9. Zero denotes a monodisperse and 9 an extremely polydisperse system.
[d] Empty liposomes.
[e] Coefficient of variation.

A summary of the characteristics of the various liposomes used is presented in Table 1.

Interaction of cis-DDP with PL bilayers

cis-DDP is reported to dissociate into positively charged Pt species in 5% mannitol (Hincall et al., 1979). The interaction between cationic Pt species, possibly present in dispersions prepared with media containing < 0.1% NaC and PL bilayers, was investigated by microelectrophoresis. If these cationic Pt species interact with the negatively charged bilayer, the negative ζ-potential of the liposomes was expected to become less negative after addition of these species to an "empty" liposome dispersion. No change was expected when *cis*-DDP is added in the neutral form (solved in 0.2% NaCl/4.2% mannitol). To study the interaction between *cis*-DDP and the PL bilayers, empty liposomes (PC/PS/Chol) prepared with 5% mannitol or 0.2% NaCl/4.2% mannitol were incubated for 30 min with 0.25 mg *cis*-DDP/ml or a saturated *cis*-DDP solution (1–1.5 mg *cis*-DDP/ml).

It was found that the ζ-potentials of the "empty" liposomes prepared with 5% mannitol and 0.2% NaCl/4.2% mannitol were −46 mV and −20 mV, respectively. Only the ζ-potential of empty liposomes prepared in 5% mannitol became less negative upon addition of *cis*-DDP (Table 2), indicating that Pt species interacted with the liposomal bilayers.

TABLE 2

Effect of cis-DDP on the ζ-potential of empty liposomes (PC/PS/Chol)

Incubation with *cis*-DDP (mg/ml) [a,b]	Liposomes prepared in	
	5% Mannitol	0.2% NaCl/ 4.2% mannitol
0	−46 mV [d]	−20 mV [d]
0.25	−24 mV	−16 mV
1.0–1.5 [c]	−17 mV	−17 mV

[a] Incubation time 30 min (25 °C).
[b] Solved as indicated for empty liposomes.
[c] Saturated *cis*-DDP solution at room temperature.
[d] Coefficients of variation of the velocities measured at the two-stationary levels for both directions were less than 15%.

In vitro tumor cell growth inhibition of cis-DDP after encapsulation in PC/PS/Chol liposomes

The tumor cell growth inhibition of *cis*-DDP encapsulated in liposomes (PC/PS/Chol) was determined by adding liposome dispersions prepared by hydration with either 0.2% NaCl/4.2% mannitol or with 5% mannitol, in different concentrations to 24 h growing monolayer cultures of 5D04

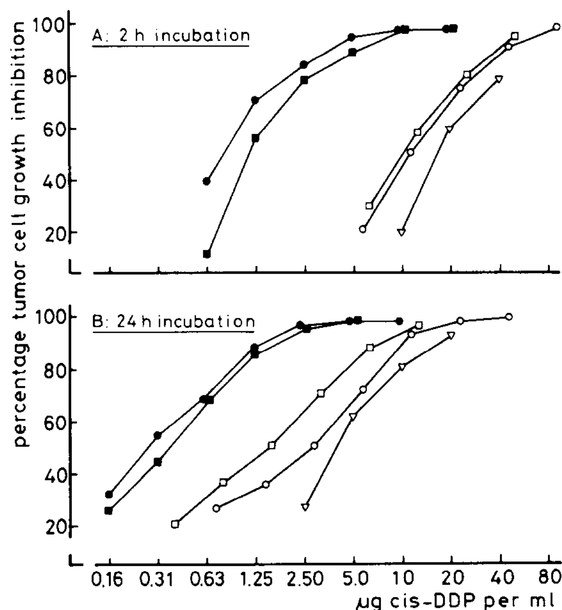

Fig. 4. Tumor cell growth inhibition in vitro of free and liposome encapsulated *cis*-DDP. Antitumor activity was determined against monolayer cultures of a murine gastric squamous cell carcinoma (5DO4). Inhibition of tumor cell growth was determined by postlabeling with [^{3}H]TdR. A: incubation period of 2 h, *cis*-DDP in: (●——●), 5% mannitol; (■——■), 0.2% NaCl/4.2% mannitol; (○——○), PC/PS/Chol liposomes in 5% mannitol: (□——□), PC/PS/Chol liposomes in 0.2% NaCl/4.2% mannitol; (▽——▽), DPPC/DPPG/Chol liposomes in 0.2% NaCl/4.2% mannitol. B: Incubation period of 24 h.

tumor cells. The tumor cell monolayers were exposed for 2, 4, 6 and 24 h. Fig. 4 represents the results after exposure of the monolayers to both free and encapsulated drug for 2 and 24 h. It was found that tumor cell growth inhibition induced by free *cis*-DDP dissolved in 0.2% NaCl/4.2% mannitol or 5% mannitol was time-dependent. After 2 h a 50% tumor cell growth inhibition was found at a concentration of approximately 1.0 μg *cis*-DDP/ml whereas after incubation during 24 h the same inhibition was found at a concentration of approximately 0.3 μg *cis*-DDP/ml. A similar time dependency was found after incubation with *cis*-DDP encapsulated in liposomes. However, at all time periods investigated tumor cell growth inhibition induced by *cis*-DDP-liposomes was approximately 4–10 times less, compared to the inhibition induced by free *cis*-DDP at equal concentration levels.

In parallel studies the stability of *cis*-DDP liposomes with respect to drug leakage in culture medium was determined. It was observed that liposomes prepared by hydration in 5% mannitol or 0.2% NaCl/4.2% mannitol were leaking only a minor part of the *cis*-DDP content during a 24-h incubation period (Table 3). The in vitro leakage test was performed under almost identical conditions as those used in the in vitro antitumor assay, however only one dilution was used (liposomes : medium = 1 : 9).

In vitro antitumor effect of cis-DDP after encapsulation in a more stable type liposome (DPPC/DPPG/Chol) (10 : 1 : 10)

The results described above indicated that the incubation of *cis*-DDP liposomes (prepared by

TABLE 3

Latency of cis-DDP liposomes during incubation of 5D04 tumor cells in tissue culture medium [a] at 37°C

Lipid composition Hydration medium	PC/PS/Chol (10 : 1 : 4) 5% mannitol	PC/PS/Chol (10 : 1 : 4) 0.2% NaCl/4.2% mannitol	DPPC/DPPG/Chol (10 : 1 : 10) 0.2% NaCl/4.2% mannitol
Latency (%) at the start of experiment	98	91	94
Latency [a,b] at t_{2h}	90 [c]	82	94
Latency at t_{24h}	86 (82)	79 (72)	95 (91)

[a] Ratio liposomes/tissue culture medium used 1 : 9 v/v.
[b] After incubation free *cis*-DDP was removed by Dowex treatment and the concentration of Pt was measured by AAS. Due to serum components, approximately 30% of the released *cis*-DDP could not be removed by the Dowex procedure, which causes an underestimation of the actual leakage. Therefore in parentheses binding percentages are given corrected for the underestimation.
[c] Mean of duplicate dispersions.

hydration with 5% mannitol or 0.2% NaCl/4.2% mannitol) in tissue culture medium during 24 h at 37 °C resulted in a slight leakage of *cis*-DDP from the liposomes. It was calculated using the corrected latency values listed in Table 3, that the amount of free *cis*-DDP present after 24 h incubation was 18 and 28% respectively of the total amount of *cis*-DDP added. To investigate whether only free *cis*-DDP was causing tumor cell growth inhibition another liposome type (DPPC/DPPG/Chol) was prepared. Based on data derived from studies with DXR-containing liposomes this "solid" liposome type was expected to be very stable against drug leakage in culture medium (submitted). The DPPG/DPPG/Chol (10 : 1 : 10) liposomes containing *cis*-DDP were prepared with 0.2% NaCl/4.2% mannitol as hydration medium. After incubation with tissue culture medium during 2 and 24 h at 37 °C it was found that no release of free *cis*-DDP had occurred (Table 3). The binding capacity of this stable type of liposome was 10.7 ± 1.2 μg *cis*-DDP/μmol phospholipid. *cis*-DDP in DPPC/DPPG/Chol liposomes showed also a long-lasting stability for more than 70 days of storage at 4–6 °C (Fig. 2). Between *cis*-DDP liposomes prepared with PC/PS/Chol (10 : 1 : 4) and with DPPC/DPPG/Chol (10 : 1 : 10) only minor differences were observed regarding their physicochemical characteristics (Table 1).

cis-DDP encapsulated in this liposome type was tested for 5D04 cell growth inhibitory activity in comparison with the free drug and the liposome type PC/PS/Chol. Fig. 4 shows that in comparison with the PC/PS/Chol liposome prepared in 0.2% NaCl/4.2% mannitol a similar tumor cell growth inhibition was induced by the DPPC/DPPG/Chol liposomes at a twofold higher concentration. After 24 h incubation almost complete tumor cell growth inhibition was found for the free *cis*-DDP at a concentration of 2.5 μg *cis*-DDP/ml while DPPC/DPPG/Chol liposomes showed an equal activity at a concentration of 20 μg *cis*-DDP/ml (Fig. 4B).

Antitumor activity in vitro of Pt after forced release from liposomes

The question was raised to what extent *cis*-DDP recovered from liposomes still possessed cytostatic properties. Therefore, after encapsulation of *cis*-DDP into liposomes (PC/PS/Chol) prepared by hydration with 0.2% NaCl/4.2% mannitol, liposomes were frozen, and thawed 3 times and subsequently ultracentrifuged at a speed of 150,000–200,000 *g* for 1 h. Supernatant was collected and the Pt concentration was determined by AAS. It was found that with this procedure 61% ± 13 ($n = 3$) of the encapsulated Pt could be recovered. When this Pt containing supernatant was tested for its tumor cell growth inhibitory activity in vitro, it was found that this supernatant was as active as free *cis*-DDP (Fig. 5). Similar results were found for *cis*-DDP liposome (PC/PS/Chol) prepared by hydration with 5% mannitol (data not shown). However, the liberation procedure of Pt from these liposomes showed only a Pt recovery of 23% ± 3 ($n = 3$).

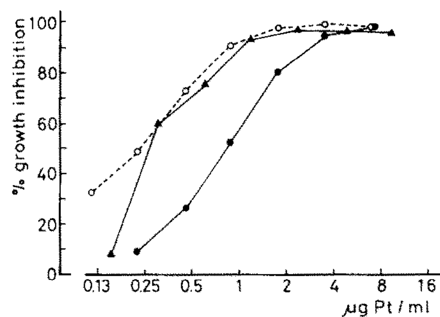

Fig. 5. In vitro antitumor effect of supernatants obtained after destruction of liposomes (three freezing/thawing cycles and subsequent ultracentrifugation) in comparison with the effect caused by free *cis*-DDP and *cis*-DDP-containing liposomes. Antitumor activity was determined against monolayer cultures of a murine gastric squamous cell carcinoma (5D04). Inhibition of tumor cell growth was determined by postlabeling with [^{3}H]TdR. Tumor cells were incubated with various drug concentrations for 24 h. (○- - -○), *cis*-DDP dissolved in 0.2% NaCl/4.2% mannitol; (●——●), *cis*-DDP in PC/PS/Chol liposomes in 0.2% NaCl/4.2% mannitol; (▲——▲), Pt recovered from liposomes by freezing and thawing (0.2% NaCl/4.2% mannitol).

Discussion

Until now, there have been only a few studies reporting the encapsulation of *cis*-DDP in

liposomes. So far, *cis*-DDP has been encapsulated in liposomes using 0.9% NaCl as hydration medium, a solution in which *cis*-DDP was found to be highly stable (Hincal et al., 1979). The low solubility of *cis*-DDP in aqueous solutions, approximately 1.0 mg/ml (Long and Repta, 1981), limits the loading capacity of liposomes. Until now, the encapsulation efficiency of *cis*-DDP containing liposomes (percentage of the amount initially added to the preparation that becomes liposome-associated) has been found to be 7.5% (Freise et al., 1982), 1.5–2.5% (Kaledin et al., 1981), 3.9% (Yatvin, 1982) and 7.5% (Sur et al., 1983). From our data it can be concluded that reduction of NaCl concentration improved the encapsulation efficiency (Table 1: 6.4% with 0.9% NaCl, 9.8% with 0.2% NaCl/4.2% mannitol and 18% with 5% mannitol). By expressing the binding of *cis*-DDP per μmol lipid (calculated from data of the previously mentioned authors) a more precise comparison can be made: 3.6 μg *cis*-DDP (Freise et al., 1982), 1.2–2.1 μg *cis*-DDP (Kaledin et al., 1981), 7.5 μg *cis*-DDP (Muzya et al. 1982) and 3.5 μg *cis*-DDP (Yatvin et al., 1982) and 45.5 μg *cis*-DDP (Sur et al., 1983) vs our results: 3.4 μg with 0.9% NaCl, 5.1 μg *cis*-DDP with 0.2% NaCl/4.2% mannitol and 9.5 μg *cis*-DDP with 5% mannitol. It must be noted that in Table 1 the binding capacity was expressed per μmol phospholipid. Considering the binding capacities obtained for dispersions prepared by hydration with 0.9% NaCl, the data calculated from results reported by Muzya et al. (1982) and Sur et al. (1983) are higher than ours. Especially the binding capacity of the multilamellar neutral liposome dispersion (PC/Chol) used by Sur et al. (1983) is extremely high. An aqueous space to lipid ratio of 4.1 μl/mg PL is reported for MLV with bilayer composition PC/PG/Chol 4 : 1 : 5 (Szoka and Papahadjopoulos, 1981). So, it can be estimated that the binding capacity of 45.5 μg *cis*-DDP/μmol lipid (75 μg *cis*-DDP/μmol PL) as reported by Sur et al., (1983) implicated that more than 90% of the liposome-bound drug must be bilayer-associated; this is rather unlikely in our view.

It is well established that the chemical stability of *cis*-DDP in aqueous solutions is very low (Hincal et al., 1979; Earhart, 1979). In the absence of Cl^- ions, *cis*-DDP will hydrolyse to cationic equation products because of replacement of the chloride ligands by water molecules. Hincal et al. (1979) showed that the addition of 5% mannitol did not prevent this instability of *cis*-DDP in water. However, addition of NaCl exhibited a significant stabilizing effect on the drug already at a concentration of 0.1%. For this reason 0.2% NaCl/4.2% mannitol was used as hydration medium in which *cis*-DDP was dissolved. The high encapsulation efficiency of *cis*-DDP in liposomes using an isotonic concentration of mannitol (5%) might be explained by the interaction of cationic Pt species with the bilayers of the liposomes. The electrostatic nature of such an interaction was indicated by the following observations (Table 2):

(1) The ζ-potential of empty liposomes (PC/PS/Chol) hydrated with 5% mannitol was -46 mV as measured by microelectrophoresis. After 30 min incubation of these empty liposomes with 0.25 mg *cis*-DDP/ml or a saturated *cis*-DDP solution dissolved in 5% mannitol the negative ζ-potential was found to be -24 and -17 mV, respectively. This effect could not be ascribed to a screening effect (data not shown). These results suggest interaction of cationic Pt species (expected to be produced by nucleophilic substitution of the chloride ligands in 5% mannitol) with the negatively charged bilayers.

(2) Indirect evidence was found by measuring the recovery of Pt from liposomes after freezing/thawing cycles. The recovery of Pt from liposomes hydrated with 5% mannitol (expected to contain positively charged Pt species) was much less ($23 \pm 3\%$) compared to the recovery of Pt from liposomes hydrated with 0.2% NaCl/4.2% mannitol (expected to contain primarily neutral *cis*-DDP molecules) ($61 \pm 13\%$).

With respect to both drug leakage (Fig. 2) and particle size the long-term stability of *cis*-DDP liposomes prepared in 5% mannitol or 0.2% NaCl/4.2% mannitol was remarkable. In contrast, *cis*-DDP liposomes (PC/PS/Chol) prepared in 0.9% NaCl were very leaky. Only Freise et al. (1982) paid some attention to drug leakage on storage. Our results generally agree with Freise et al. (1982) who observed a Pt release of 25% from

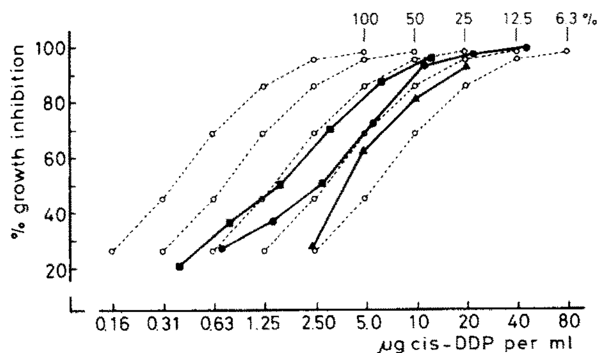

Fig. 6. Estimated antitumor activity of *cis*-DDP liposomes based on drug leakage. It is assumed that *cis*-DDP containing liposomal entities do not exert a cytostatic activity on their own, but only show antitumor activity after releasing their *cis*-DDP content. From the free drug curve depicted in Fig. 4B the tumor growth inhibition in vitro was calculated if only 50, 25, 12.5 and 6.3% (Pt) was released from *cis*-DDP liposomes during 24 h incubation with 5D04 tumor cells (○---○). The curve of the tumor growth inhibition of *cis*-DDP liposomes (PC/PS/Chol) in 0.2% NaCl/4.2% mannitol fits about 25% (■——■); *cis*-DDP liposomes (PC/PS/Chol) in 5% mannitol fits about 12.5% (●——●) and *cis*-DDP liposomes (DPPC/DPPG/Chol) in 0.2% NaCl/4.2% mannitol fits between 6.3 and 12.5% (▲——▲).

liposomes dispersed in 0.9% NaCl already after two days of storage. Therefore it was studied whether the presence of mannitol influenced the stability of *cis*-DDP liposomes against *cis*-DDP leakage. Addition of 4.2% mannitol to 0.9% NaCl hydration medium did not reduce the leakage rate. The mechanism behind the difference in drug release on storage between *cis*-DDP liposomes hydrated with 0.9% NaCl and liposomes hydrated with 0.2% NaCl/4.2% mannitol is still obscure. Also comparison of the various liposomal characteristics as presented in Table 1 does not give any explanation.

The tumor cell growth inhibition in vitro of *cis*-DDP containing liposomes seems to be dependent on the leakage of *cis*-DDP from liposomes into the tissue culture medium (Fig. 4). Parallel studies on the latency of *cis*-DDP liposomes (PC/PS/Chol) in tissue culture medium at 37 °C revealed that after 24 h approximately 28% of the *cis*-DDP was not associated with liposomes prepared with 0.2% NaCl/4.2% mannitol (Table 3).

In Fig. 6 growth inhibition curves of hypothetic *cis*-DDP liposome dispersions with different leakage rates in tissue culture medium are drawn calculated from the free drug curve depicted in Fig. 4B assuming that all cytostatic effects observed were exclusively caused by free *cis*-DDP present in the tissue culture medium. It then appears that the growth inhibition curve measured for liposomes (PC/PS/Chol) hydrated with 0.2% NaCl/4.2% mannitol (Fig. 4B) corresponds to a hypothetic curve depicted for the action of 25% free *cis*-DDP. This value of 25% is in good agreement with the 28% free drug measured in case of the 24 h in vitro incubation in tissue culture medium (Table 3). By analogy, the growth inhibition curve measured for liposomes (PC/PS/Chol) hydrated with 5% mannitol (Fig. 4B) corresponds to a curve estimated for the action of 12.5% free *cis*-DDP, which is in reasonable agreement with the 18% free drug measured after a 24 h in vitro incubation in tissue culture medium (Table 3). The growth inhibition curve for DPPC/DPPG/Chol liposomes is located between the 6.3% and 12.5% free-drug curves. This finding correlates well with the 9% free drug present after a 24-h in vitro incubation (Table 3). This correlation between the amount of non-liposome-associated Pt after incubation in tissue culture medium (Table 3) and the calculated growth inhibition curves (Fig. 6) strongly suggests that the different liposomal antitumor effects observed were caused by differences in liposomal stability with respect to loss of *cis*-DDP content. This observation is in agreement with others (Allen et al., 1981, Renswoude and Hoekstra, 1981, Kercret et al., 1983), who have demonstrated that the antitumor activity in vitro was due to drug which has leaked out of the liposomes. Our data do not suggest a direct uptake of drug in the liposomal form by our tumor cells. If such an association would have occurred, an additional antitumor activity compared to the antitumor activity of free cis-DDP is expected for *cis*-DDP liposomes.

Our data indicate that the resistance against drug leakage of *cis*-DDP liposomes (PC/PS/Chol) in tissue culture medium at 37 °C was much higher than that observed for the same liposome type containing DXR (G. Storm et al., manuscript in preparation). With the same tumor cell line (5D04)

we found no difference in tumor cell growth inhibition between free DXR and DXR encapsulated in liposomes, indicating a relatively large DXR release during the incubation period (G. Storm et al., submitted). Since it is reported that the more amphiphilic DXR strongly interacts with liposomal membranes (Crommelin et al., 1983), this finding again emphasizes the critical importance of the physicochemical nature of the drug for the degree of drug leakage.

Although the in vitro measurement of tumor cell growth inhibition for *cis*-DDP liposomes may have limited relevance for their behavior in vivo, our experiments have shown that encapsulated *cis*-DDP present in the liposomes as intact *cis*-DDP molecules (0.2% NaCl/4.2% mannitol) or as cationic Pt equation products (5% mannitol), still has antitumor potency after leakage (Fig. 4) or forced liberation (Fig. 5) from the liposome structure. This could be an important observation since it has been shown that after intravenous injection the majority of the liposomes will be entrapped by the cells of the reticuloendothelial system (RES), especially in liver and spleen (Poste et al., 1984, Weinstein and Leserman, 1984). After this entrapment, liposomes may be degraded with subsequent release of encapsulated drug (G. Storm et al., submitted; Scherphof et al., 1983). As *cis*-DDP can be recovered from liposomes without losing its antitumor activity in vitro (Fig. 5) one may speculate that degradation of *cis*-DDP liposomes by cells of the RES may result in a slow release of active Pt species. Therefore, additional studies to the in vivo antitumor activity of *cis*-DDP liposomes are in progress.

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