*Eur J Cancer Clin Oncol*, Vol. 22, No. 10, pp. 1139–1147, 1986.
Printed in Great Britain

0277-5379/86$3.00+0.00
Pergamon Journals Ltd.

# Effects of *Cis*-diamminedichloroplatinum (II) Loaded Liposomes on Mouse Ehrlich Tumor Cells*

MARIE-CLAIRE A.J. DE PAUW-GILLET, ERNST HEINEN, GEORGES Y.J. WEBER and ROGER J.B. BASSLEER†

*Department of Histology and Cytology, University of Liège, Rue de Pitteurs 20, B-4020 Liège, Belgium*

**Abstract**—Cis-*diamminedichloroplatinum II (cisplatin) heavily or lightly loaded (fluid, solid, negatively charged or neutral) liposomes were prepared. Cisplatin release from liposomes was observed only after long dialysis times or after liver lysosomal enzymatic disintegration in solution. Mouse Ehrlich tumor cells (ELT) cultured* in vitro *were treated with cisplatin, liposomes or cisplatin loaded liposomes, and the effects on the mitotic activity, the DNA content and the ultrastructure were compared. Cisplatin* (1–10 μg/ml) *had an antimitotic activity and modified the DNA content in ELT cells. Ribosome aggregation, perichromatin or interchromatin granule accumulation, and chromatin condensation or some degree of dispersion could be observed.*

*Negatively charged fluid liposomes had an antimitotic activity and modified the DNA content in ELT cells at lower concentrations* (0.3 μmoles/ml) *than in the case of neutral fluid liposomes* (1.5 μmoles/ml). *Negatively charged solid liposomes were not toxic at these concentrations. Ultrastructural analysis of ELT cells treated* in vitro *with negatively charged fluid liposomes revealed their extracellular adsorption and their disintegration in phagolysosomes. A fusion between liposomes and the plasma membrane was not definitely demonstrated. Cisplatin loaded liposomes also had an antimitotic activity and modified the DNA content in ELT cells. These effects were similar to or more pronounced than those induced by free cisplatin. Ultrastructural analysis revealed some kind of electron dense material in phagolysosomes which was never observed after the treatment with free cisplatin or liposomes alone. Effects on nucleic acids were rarely observed.*

## INTRODUCTION

CISPLATIN is a potent anticancer agent, the usefulness of which is limited by its toxicity [1–4]. This is why attempts have been made to incorporate it in pharmacological vectors targeted at tumor cells, such as liposomes [5–11].

We present here results concerning cisplatin incorporation into different types of liposomes, and their effects on mouse Ehrlich tumor tetraploid cells (ELT) cultured *in vitro*. Mitotic activity, DNA content and, in the case of negatively charged fluid liposomes, ultrastructure were analyzed. We also provide new evidences concerning the mechanisms of cisplatin action on cells and DNA.

Accepted 2 December 1985.
*Work supported by: Fonds de la Recherche Scientifique Médicale, Centre Anticancéreux près l'Université de Liège, and Association Sportive contre le Cancer (Belgium).
†To whom all requests for reprints should be addressed.

## MATERIALS AND METHODS

### *Cisplatin*

Cisplatin was obtained from Polysciences. Two methods were used for measuring its concentration: a colorimetric reaction with orthophenylenediammine (OPDA) in dimethylformamide, as suggested for metallic platinum [12], but modified for cisplatin [7] and X-ray emission induced by protons (P.I.X.E.; [13]). Stock solutions were prepared by dissolving cisplatin in distilled water (24 hr at room temperature with agitation in the dark).

### *Liposome preparation*

Dipalmitoylglycerophosphocholine (DPPC); L-alpha phosphatidic acid (PA); L-alpha-phosphatidylcholine or lecithin (PC), stearylamine (SA) and cholesterol (CHOL) were obtained from Sigma

*Table* 1. *Type of liposomes used and molar ratios of constitutive lipids*

| Molar ratios | Solid liposomes | | | Fluid liposomes | | |
|---|---|---|---|---|---|---|
| | Positive ($S^+$) | Negative ($S^-$) | Neutral ($S^0$) | Positive ($F^+$) | Negative ($F^-$) | Neutral ($F^0$) |
| 7 | DPPC | | | PC | | |
| 2 | CHOL | | | CHOL | | |
| 1 | SA | PA | CHOL | SA | PA | CHOL |

(powders) or from Lipid Products (chloroform–methanol solutions).

Sterile conditions were maintained during all preparation steps. Appropriate lipid compounds (Table 1) were dissolved in chloroform, and evaporated under nitrogen. The thin film of lipids formed on the glass wall was then suspended in an aqueous buffer solution (5 ml of phosphate 6 mM, NaCl 1 mM, pH 7.2) by agitation (Vortex) at room temperature (for fluid liposomes) or at 60° C (for solid ones).

After 1 hr incubation at room temperature, multilamellar liposomes were sonicated for 1–20 min in order to obtain homogeneous suspensions of either multilamellar liposomes (MLV; short sonication times) or mostly unilamellar ones (SUV; long times of sonic dissociation).

For preparing cisplatin loaded liposomes, 1000 or 5000 μg of cisplatin from stock solutions were incorporated into 5 ml of buffer solution before agitation, to lipid concentrations of $0.129 \times 10^{-4}$ or $0.645 \times 10^{-4}$ mol/5ml. Suspensions were maintained at room temperature from 1–24 hr before chromatography on a Sepharose 4 B column (30 × 1.5 cm). The solutions of cisplatin alone in buffer medium used as controls were kept under the same conditions. Fractions containing liposomes (quantitative estimation by turbidity measurements at 300 nm) and cisplatin (measurements by P.I.X.E. or OPDA method) were kept at 4° C and used within 1 day.

*Enzymatic degradation*

Cisplatin loaded liposomes were incubated with an extract of liver lysosomal hydrolases (furnished by Dr P.Tulkens, from Institute of Cellular Pathology, Université Catholique de Louvain, at Woluwé, Belgium), in a buffer solution: 0.05 M acetate (pH 7.4) with $9.10^{-4}$M $MgCl_2$, in NaCl 0.1 M, at 25 or 37° C. Cisplatin release as a function of time was measured by OPDA assay.

*Ehrlich tumor cell cultures*

ELT cells (tetraploid line) were obtained from the peritoneal cavities of tumor bearing C 57 Black 6 J mice. Cell suspensions were spread, after washing, on glass slides in Petri dishes containing 2 ml of culture medium: NCTC 109 (45%), Hanks liquid (50%), foetal calf serum (5%) and 100 IU penicillin per ml. Culture dishes were incubated at 37° C in 95% air, 5% $CO_2$ humidified atmosphere. The culture medium was renewed every day.

*Experimental*

The ELT cells were treated for 1–48 hr by adding to the culture medium one of the following agents: cisplatin alone (0.5, 5 or 10 μg/ml); cisplatin incorporated into solid or fluid liposomes, either negatively charged or neutral (0.5, 1 or 2 μg and 0.05, 0.01 or 0.02 × $10^{-6}$ mol/ml); and liposomes alone (from 0.5 to 3 μmol/ml).

*Optical microscopy*

Cells cultured on glass slides were fixed in a mixture acetone–absolute ethanol (1 : 1) for the Feulgen reaction or in formol–calcium 10% for lipid staining (Red Oil).

The mitotic index was established on 3000 cells for each slide (two slides in each experiment); differences were evaluated statistically by the Student's *t*-test.

Nuclear DNA content was measured by a scanning and integrating (Vickers M 86) microdensitometer at 540 nm. Fifty individual nuclei were measured on each slide (two slides in each experiment). The 2 C (diploid) base line was calculated from mouse leucocytes on the same slides. Comparison of frequency histograms composed of four classes (2 C, intermediate, 4 C and polyploid nuclei) was performed by Pearson's corrected $\chi^2$ test.

*Electron microscopy*

A transmission electron microscope (Philips 301) was used (at 80 kV) for the ultrastructural analysis of ELT cells cultured *in vitro* and liposome suspensions.

Cells were fixed for 30 min in glutaraldehyde 2.5% solution buffered with sodium cacodylate 0.2 M at pH 7 and thereafter with $OsO_4$ and included in Epon 812. In some cases, thiocarbohydrazide postfixation [14] was performed and followed by uranyl acetate contrast and rapid inclusion in Epon [15].

For scanning electron microscopy, cultured cells were rinsed in Hanks fluid before fixation in glutaraldehyde 2.5% and postfixation with $OsO_4$. Pieces were dehydrated using the $CO_2$ critical point technique and gold metallized before examination under a Siemens Etec microscope, at 20 kV.

## RESULTS

*Liposomes preparation*

Liposomes of different compositions (see Table 1) were prepared. Positively charged liposomes were not used here, because they were very toxic for ELT cells cultured *in vitro*: cytotoxic effects were already noted after 4 hr of treatment with low lipid concentrations (0.5 μmol/ml culture medium) and severe cellular vacuolization was observed for concentrations at which negatively charged or neutral liposomes were not cytotoxic.

The method of platinum determination (by OPDA or by P.I.X.E.), the period of time separating the preparation of liposome suspensions and column chromatography, the duration of sonication and also the lipid components used were factors determining the quantity of cisplatin incorporated into liposomes (see Table 2). We observed that a high concentration of cisplatin in the buffered suspension before chromatography, a longer incubation time or a shorter sonication time were better for obtaining high cisplatin incorporation into the aqueous liposome phase. Solid liposomes incorporated more cisplatin than fluid liposomes did; negatively charged solid liposomes incorporated more than neutral solid ones did. Fifty per cent of the initial quantity of cisplatin present in the buffered suspension before chromatography could be associated with neutral or negatively charged solid liposomes (even with long sonication time, 15 min). Only 10% could be incorporated into neutral or negatively charged fluid liposomes, and only if short sonication times were used (≤ 3 min).

Dialysis of multilamellar negatively charged fluid or solid liposomes for 24 hr at 25° C with a phosphate buffer or with physiological saline solution (Tyrode glycosol solution) did not result in release of cisplatin for the first 48 hr. Four days later, about 9% of the incorporated cisplatin in buffered solutions and 24% in physiological solutions were released from solid liposomes, and about 17 and 30% from fluid liposomes, respectively.

An extract containing liver lysosomal hydrolases disintegrated negatively charged fluid liposomes, with about 10% release of cisplatin from liposomes, at 25° C or at 37° C.

Multilamellar liposomes were analyzed under a transmission electron microscope without any additional contrast when cisplatin was incorporated (Fig. 1).

*Table 2. Quantity of cisplatin* (μg/ml) *incorporated into liposomes as measured in fractions collected after chromatography on Sepharose* 4 B *columns*

I. Mixture of 100 μg of cisplatin and 3 μmol of lipids per ml of buffer solution; 15 min sonication before incubation at 25°C (in the dark) for different lengths of time; detection of cisplatin in liposomes after chromatography by O.P.D.A. reaction.

| Time at 25° C | Cisplatin (μg/ml) | |
|---|---|---|
| | $F^-$ | $S^-$ |
| 1 hr | 0 | 2 |
| 20 hr | 10 | 20.4 |

II. Mixtures of 3 (A) or 30 (B) μmoles of lipids and 100 or 1000 μg of cisplatin per ml of buffer solution; 15 min sonication before incubation at 25°C for 20 hr (or 40 hr*); detection of cisplatin in liposomes after chromatography by O.P.D.A. reaction.

| | Cisplatin (μg/ml) | | | |
|---|---|---|---|---|
| *Before chromatography* | *After chromatography* | | | |
| | $F^-$ | $S^-$ | $F^0$ | $S^0$ |
| 100 (A) | 10 | 20 | 10 | 20.4 |
| 1000 (B) | 10 | 100 | 10 | 60 |
| | | | | 100 (*) |

III. Mixtures of 100 μg of cisplatin and 30 μmoles of lipids per ml buffer solution; 15 min sonication (or 1 min**), 20 hr at 25°C (or 40 hr*).

| | Cisplatin (μg/ml) | | | |
|---|---|---|---|---|
| Method | $F^-$ | $S^-$ | $F^0$ | $S^0$ |
| O.P.D.A. | 10 | 100 | 10 | 100(*) |
| | 10(**) | | | |
| P.I.X.E. | 10 | 190 | 10 | 370(*) |
| | 100(**) | | 100(**) | |

*Cytological, cytochemical and ultrastructural analysis of ELT cells cultured* in vitro *and treated with cisplatin alone*

When treated with cisplatin, ELT cells always showed mitotic inhibition ($P < 0.001$; Table 3) and very often alterations of the DNA content. The accumulation of nuclei either with 4 DNA, 8 DNA or highly polyploid cells, according to experimental conditions, is well documented [16]. Ultrastructural analysis of ELT cells treated with cispla-

*Table 3. Mitotic activity in ELT cells cultured* in vitro *and treated for* 24 hr *with cisplatin* (1 μg/ml) *alone or incorporated into different types of liposomes*

| | Mitotic Index (‰) | | |
|---|---|---|---|
| Liposomes (μmol/ml) | | Cisplatin | |
| sonication time | Controls | Free | Inside liposomes |
| $S^-$ (≤ $10^{-5}$); 15 min | 5 | 0 | 0 |
| $F^-$ (≤ 1); 15 min | 9 | 0.5 | 0 |
| $F^-$ (≤ 0.05); 1 min | 21 | 6 | 6 |
| $F^0$ (≤ 0.4); 10 min | 14 | 5 | 4 |
| $F^0$ (≤ 0.4); 20 min | 17 | 0.5 | 0 |

tin (1 μg/ml for 24 hr) showed chromatin condensation, perichromatin and interchromatin granule accumulation, (Fig. 2a) and, in some cases, compact nucleoli. Ribosome aggregates in a helicoidal disposition were frequently noted in the cytoplasm (Fig. 2b); but their number was quite variable. After treatment with 10 μg cisplatin for 1 hr, transparent vacuoles, lipid droplets and phagolysosomes with electron-dense material could also be observed in the cytoplasm (Fig. 3b). In some nuclei, amorphous material which could be skeletal was seen in the nucleoplasm (Fig. 3a).

*Cytological, cytochemical and ultrastructural analysis of ELT cells cultured* in vitro *and treated with liposomes alone*

Negatively charged solid liposomes (15 min sonication; 0.1 or 1 mM final concentration in culture medium) had little or no effects on ELT cells when treated for 24 hr, as far as mitotic activity, DNA content, and cellular degeneration were concerned.

Negatively charged fluid liposomes (15 min sonication; 1 mM final concentration in culture medium) significantly inhibited ($P < 0.001$) mitotic activity ($4^{o}/_{oo}$ as opposed to $9^{o}/_{oo}$ in controls) and significantly increased ($P < 0.001$) 4 DNA cell percentages (84 vs. 54% in controls), suggesting arrest in G 1 after a 24 hr treatment. Nuclei were frequently observed with a DNA content lower than 4 DNA.

Neutral fluid liposomes (15 min sonication; 1.5 mM final concentration in culture medium) significantly inhibited ($P < 0.001$) mitotic activity ($5^{o}/_{oo}$ vs. $17^{o}/_{oo}$ in controls) and significantly increased ($P < 0.001$) 4 DNA cell percentages (88 vs. 58% in controls). Nuclei with less than 4 DNA were also observed following a 24 hr treatment.

Ultrastructural analysis of ELT cells treated with negatively charged fluid liposomes (3 min sonication; 0.1 mM, during 24 hr) revealed adsorption of the liposomes on the cell surface (Fig. 4). Their disintegration took place either inside vacuoles, whose number increased rapidly (Fig. 5a) or, in some cases, externally. We observed few microvilli but numerous blebs which could perhaps be related to fusion of liposomes with the plasma membrane or to cytoplasmic expansions (Fig. 5b). Mitochondrial alterations were also observed (Fig. 5c).

*Cytological, cytochemical and ultrastructural analysis of ELT cells cultured* in vitro *and treated with cisplatin loaded liposomes*

Cisplatin heavily loaded negatively charged solid liposomes were obtained after 15 min sonication; 1 ml of suspension stock contained about $2 \times 10^{-3}$ μmol lipid (estimation by turbidity measurements at 300 nm) and 100 μg cisplatin (P.I.X.E. measurements). ELT cells were treated in culture with 0.5 or 1 μg/ml cisplatin incorporated into negatively charged solid liposomes. With either concentrations the effects obtained with free cisplatin and cisplatin loaded liposomes were identical for the same final concentration of cisplatin in the culture medium: i.e. mitotic inhibition (Table 3) and/or DNA content alteration. At these liposome concentrations, lipid toxicity was negligible.

Negatively charged fluid liposomes were found to be heavily cisplatin loaded after 1 min sonication or lightly cisplatin loaded after 15 min sonication. Mitotic inhibition by these liposomes was similar to that due to cisplatin alone in both cases. Similar results were obtained with neutral fluid liposomes highly loaded with cisplatin (Table 3). A dramatic increase in $\leqslant 4$ DNA cells was observed with lightly loaded liposomes ($F^-$ or $F^o$; from $62 \pm 10\%$ in controls to $98 \pm 2\%$ in treated ones).

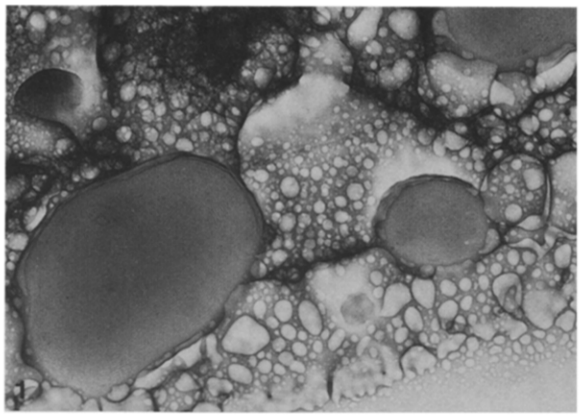
Ultrastructual analysis was performed on ELT cells treated with cisplatin heavily loaded negatively charged fluid liposomes. This type of liposomes is probably the only one able to fuse with the plasma membrane *in vitro* [17] and would be able to deliver its content into the cytoplasm. Adsorption of cisplatin loaded $F^-$ liposomes on the surface of ELT cells was observed under scanning electron microscopy. Under transmission electron microscopy, dense material was detected inside small cytoplasmic vacuoles which were probably phagolysosomes loaded with degraded lipids and/or with cisplatin. Cisplatin alone or liposomes alone did not show similar dense material (Fig. 6a). Ribosome aggregates in a helicoidal disposition were not observed; but lipid droplets, cytoplasmic vacuolization and, in rare cases, perichromatin granule accumulation were noted in treated cells.

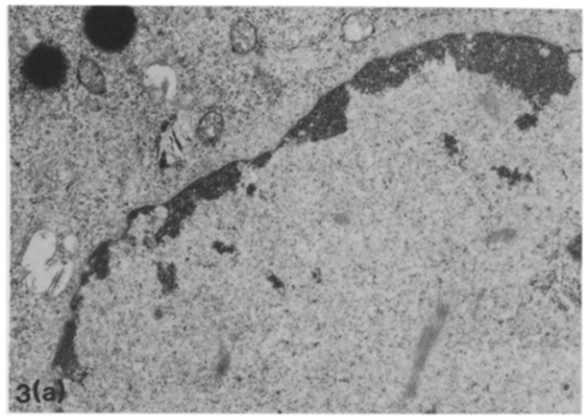
After post-fixation with thiocarbohydrazide as suggested by Raz *et al.* [14], another fact was observed but only in cells treated with cisplatin loaded liposomes. Elongated lamellar or round elements with homogeneous electron dense material were detected outside the cells, close to the plasma membrane (Fig. 6b). In some cases, similar dense material was present inside dilated microvilli (Fig. 6c). Degraded material either adhered to cell surfaces or was found inside cytoplasmic vacuoles.

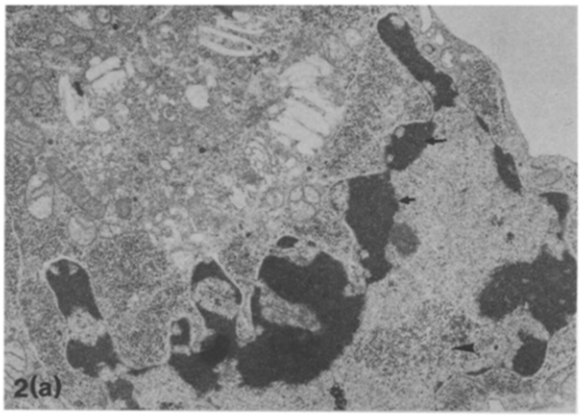
## DISCUSSION

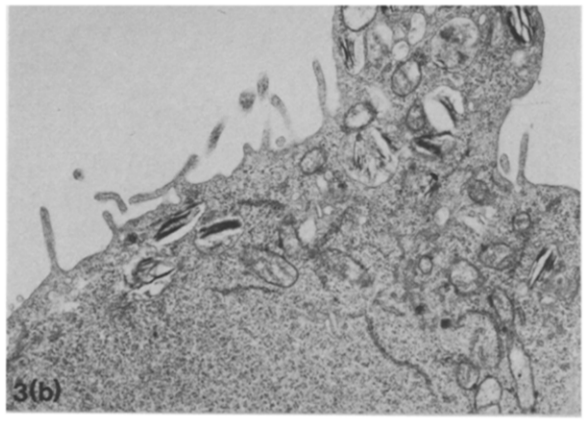
*Preparation and characterization of cisplatin loaded liposomes*

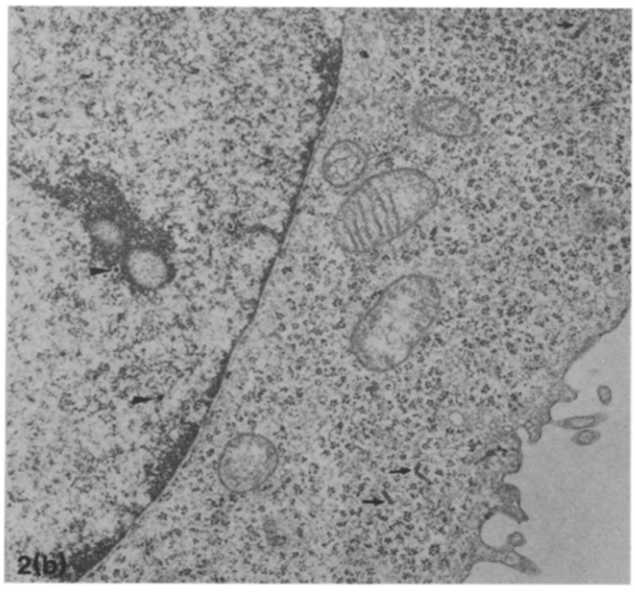
Relatively high concentrations (50%) of cisplatin could be incorporated into liposomes, when suspensions were maintained for 24 hr at room tem-





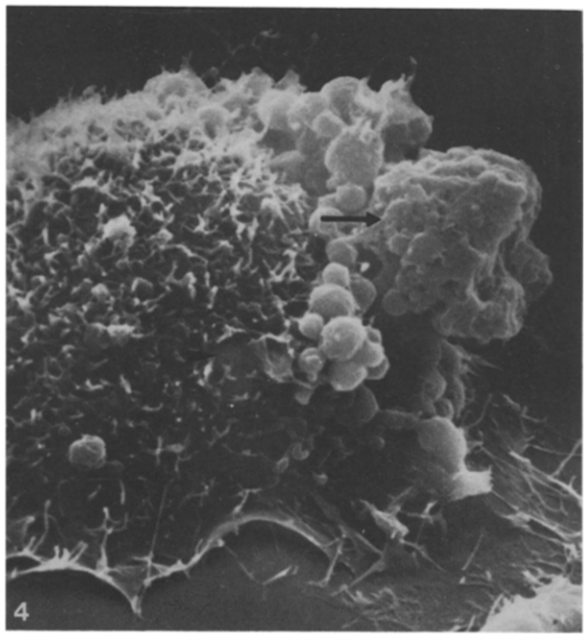




*Fig.* 1. *Transmission electron microscope observation of neutral fluid liposomes loaded with cisplatin*; 20 min *sonication. Non-contrasted preparation. Liposomes were multilamellar; their contrast was due to the presence of cisplatin.* (× 85,000).

*Fig.* 2. *Transmission electron microscope examination of ELT cells treated* in vitro *with cisplatin* (1 μg/ml) *during* 24 hr. (a). *Condensed chromatin areas* (→) *were located close to the nuclear envelope. Interchromatin granules were also observed* (➔). (× 7600). (*b*) *Some ribosome aggregates in helicoidal disposition were observed in the cytoplasm* (→). *In the nucleus* (*on the left side of the picture*), *some perichromatin granules were visible* (➔). (× 14,800).

*Fig.* 3. *Transmission electron microscope observation of ELT cells treated* in vitro *with cisplatin* (10 μg/ml) *during* 1 hr. (a) *In the nucleus, filamentous material* (*apparently skeletal*) *was found.* (× 16,380). (b) *Cytoplasmic vacuoles contained some dense material which could well be cisplatin accumulated inside phagolysosomes.* (× 16,835).

*Fig.* 4. *Scanning electron microscope observation of ELT cells treated* in vitro *with negatively charged fluid liposomes* (0.1 mM), 3 min *sonication, during* 24 hr. *Numerous small liposomes were in contact with the ELT cell surface; one large irregular round structure* (→) *probably corresponded to some disintegrated liposomes outside the cell; the local portion of cell surface devoid of microvilli* (➔) *could be due to ongoing fusion of a liposome.* (× 2940).

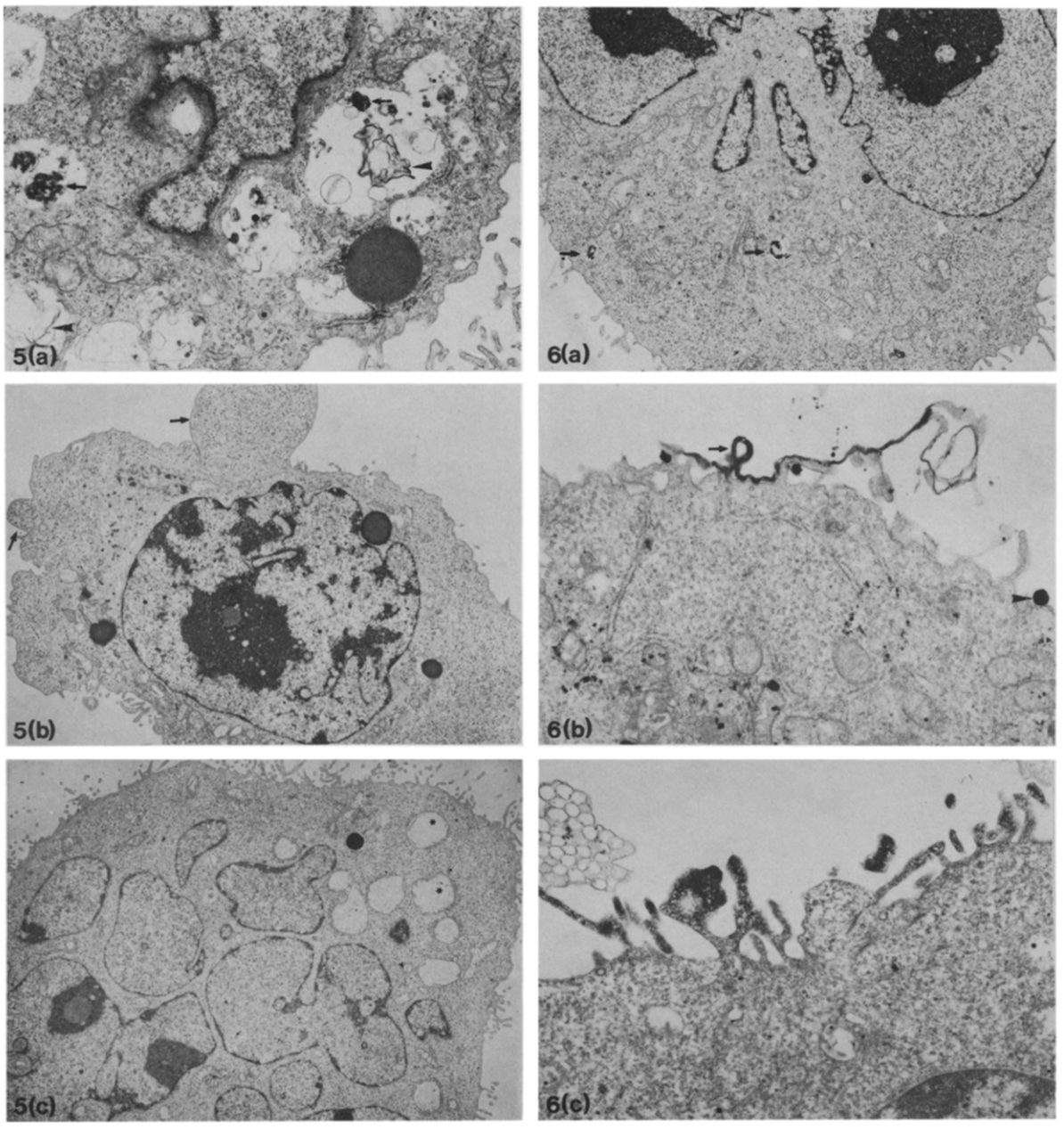



*Fig.* 5. *Transmission electron microscope observation of ELT cells treated* in vitro *with negatively charged fluid liposomes* (3 min *sonication*). (a) 0.75 mM, *during* 1 hr, *post-fixation with thiocarbohydrazide, according to Raz* et al. [6]. *Homogeneous* (→) *or lamellar* (➔) *dense material was observed inside cytoplasmic vacuoles* (× 9600). (b) *and* (c) 0.1 mM *during* 24 hr; (b) *blebs of cytoplasm expansions were frequently noted* (→). (× 2.400). (c) *mitochondria were swollen* (★) *in a multinucleate giant cell* (× 2400).

*Fig.* 6. *Transmission electron microscope observation of ELT cells treated* in vitro *with cisplatin loaded negatively charged fluid liposomes* (1 min *sonication*). (a) 0.003 μmoles *of lipid and* 1 μg *of cisplatin*/ml, *during* 24 hr. *Dense material was present in numerous cytoplasmic vacuoles* (→) (× 3.400). (b) *and* (c) 0.03 μmoles *of lipid and* 10 μg *of cisplatin*/ml *during* 1 hr. *Thiocarbohydrazide post-fixation.* (b) *Homogeneous very dense material was associated to lamellar* (→) *or round* (➔) *structures at the surface of the cell and could well be liposomes* (× 13.600). (c) *Microvilli contained dense material which could originate from cisplatin loaded liposomes* (×13.600).

perature. Yatvin *et al.* [10] had also observed a better cisplatin incorporation into liposomes under similar conditions. However, only 4% cisplatin was detected in solid liposomes (radioactivity measurements with 195 Pt). Attention must be drawn to the methodology used for Pt detection. The OPDA method described by Golla and Ayres [12] did not seem to be adequate for Pt measurements, at least under our experiments.

Dialysis experiments indicated that cisplatin was not released from liposomes during the first 48 hr at room temperature, but disintegration with an extract of liver lysosomal hydrolases showed a partial cisplatin release (about 10%) from the liposomes. Thus, in contrast to vectors for cisplatin transport such as DNA [6, 7], liposomes partially released cisplatin in a free state both under lysosomal extract disintegration, and under dialysis (though after a longer period of time). It could be postulated that cisplatin loaded liposomes penetrated by endocytosis into the cell, lysosomes fused with phagosomes (containing the drug-vector complex) and probably induced a release of cisplatin which could then leave the vacuole in a free state and reach the cytoplasm.

*Effects of cisplatin alone on ELT cells cultured* in vitro

Mitotic activity was always lowered, independently of its level observed in controls.

Alterations of the DNA content in ELT cells cultured *in vitro* and treated with cisplatin alone were quite variable. This was probably due to the fact that G 1 cells were more sensitive to this agent than G 2 or S cells [18]. So, if the treatment started with populations in which G 1 cells were majoritary, the effects on DNA were more intense.

At the ultrastructural level, nuclear and cytoplasmic alterations were essentially due to the covalent fixation of cisplatin on the nucleic acids. Ribosome aggregates were similar to those observed in plant cells treated with cisplatin [19]; they were observed in sarcoma 180 cells [20]. Chromatin condensation, perichromatin and interchromatin granule accumulation observed in ELT cells treated with cisplatin have been described in normal liver or kidney cells from rats treated with cisplatin. They have been attributed to intra- or intercrosslinks of cisplatin with DNA in chromatin [21]. The complete dispersion of condensed chromatin was not observed in treated ELT cells as it was observed in plant cells after the treatment with cisplatin [19]. Perhaps the quantity of cisplatin fixed to the chromatin was different for animal and plant cells. Some dispersion of chromatin was however noted in treated ELT cells; this could be related to the observation of "clear chromatin" in sarcoma 180 cells treated with cisplatin [20].

Tentatively, we propose to assign the chromatin dispersion in cisplatin treated ELT cells to covalent bonds which open the secondary structure of DNA, i.e. bifunctional crosslinks of one cisplatin molecule on two N–7 of adjacent guanine residues; this opening was confirmed at low levels of cisplatin fixed on isolated chromatin or nucleosomes in solution [22]. Such a covalent bond could allow some RNA synthesis to occur, as suggested by nucleolar and ribosomal ultrastructure, as well as some DNA synthesis, as shown by our DNA contents measurements. The chromatin condensation of cisplatin treated ELT cells could be tentatively related to aggregation phenomena observed in solutions of isolated chromatin or nucleosomes treated with high cisplatin level [22]. In this case, intermolecular crosslinks essentially between guanine residues and DNA-protein crosslinks could be responsible for such an aggregation [22–24]. Molecular bonds of these latter types could inhibit RNA synthesis [25].

*Effects of liposomes alone or loaded with cisplatin in ELT cells*

Some of our results agree with those that Behrens *et al.* [26] obtained on human cells or rat kidney cancer cells cultured *in vitro*. By measuring cell survival, the cytotoxicity of liposomes appeared to be modulated by the lipid concentration, the type of liposome and also the sonication time. Generally, positively charged liposomes were very toxic, negatively charged liposomes were more toxic than neutral liposomes, and fluid liposomes were more toxic than solid liposomes. Negatively charged fluid liposomes were the most toxic; this fact could be related to the liposome fusion with the plasma membrane, as postulated by other authors for cultured mammalian cells [27, 28]. Adsorption and intracytoplasmic disintegration of liposomes by ELT cells cultured *in vitro* was observed in the case of negatively charged fluid liposomes.

Increased cytotoxicity of cisplatin loaded liposomes could be achieved *in vitro*, for the same cisplatin concentration, only when the liposomes were not heavily loaded, probably because under these conditions, a lipid concentration toxic for the cells was added. In this respect, our results agree with others describing EMT 6 cell survival after treatments with liposomes loaded with methotrexate, actinomycin D or cytosine 1-3-D-arabinofuranoside [29]. Ultrastructural analysis of ELT cells cultured *in vitro* and treated with cisplatin loaded negatively charged fluid liposomes showed that they were adsorbed on the cell surface or penetrated into cytoplasmic vacuoles, where they were progressively degraded. Their fusion with the

plasma membrane was also likely but we had no definitive evidence. We rarely observed ultrastructural modifications related to nucleic acids, as it was the case for ELT cells treated with free cisplatin in culture medium.

These results suggest that cisplatin is not released from liposomes into the medium, at least, not in large quantities, as postulated for methotrexate [29]. Interestingly, methotrexate is exclusively incorporated into the liposome aqueous phase, while cisplatin is distributed between aqueous and lipid phases [10].

Results obtained with another drug, *auranofin*, a gold non-polar compound, showed variations in the cellular toxicity after its incorporation into the liposome lipid phase. In cultured human synovial cells [30], the toxicity of liposome-bound auranofin was much lower than that of free auranofin. It has been demonstrated that this agent was inactive when present in lipid cell compartments [31].

An analogy may be made for our experiments. Perhaps, the lipid part of total cisplatin content is inactivated in a similar way. We cannot exclude that some variations in active cisplatin concentrations resulted from the conditions of liposomes preparation.

## REFERENCES


1. Macquet J, Butour J, Johnson N. Physicochemical and structural studies on the *in vitro* interactions between Pt (II) compounds and DNA. *ACS Symp Ser* 1983, **209**, 75–100.
2. Hitchock A, Lock C, Pratt W, Lippert B. Platinum complexes with DNA bases, nucleotides, and DNA. *ACS Symp Ser* 1983, **209**, 209–227.
3. Heinen E, Bassleer R. Cytological and cytochemical analysis of the effects of cis-dichlorodiamminoplatinum (II) on chick fibroblasts cultivated *in vitro*. *Chemother* 1976, **22**, 253–261.
4. Heinen E, Bassleer R. Mode of action of cis-dichlorodiammine platinum (II) on mouse Ehrlich ascites tumour cells. *Biochem Pharmacol* 1976, **25**, 1871–1875.
5. de Duve C. Lysosomes and chemotherapy. In: Harris R, ed. *Biological Approaches to Cancer Chemotherapy*. London, Academic Press, 1961, 101–112.
6. Heinen E, Desaive Cl, Houssier Cl, Gillet M, Chèvremont M. Effets du complexe cis-dichloro-diammino-platine (II) — acide désoxyribonucléique sur des cellules normales ou cancéreuses. *C R Soc Biol* 1976, **170**, 919–921.
7. De Pauw-Gillet M, Houssier Cl, Fredericq E. Interaction of DNA and purine nucleosides with cis-dichlorodiammineplatine (II) and antimitotic activity of the complexes on meristematic root cells. *Chem Biol Interact* 1979, **25**, 87–102.
8. Gregoriadis G, Senior J, Trouet A, eds. "Targeting of drugs" NATO Advanced Study Institutes. Ser A. *Life Sci* 1982, **47**.
9. Kaledin V, Matienko N, Budker V, Nikolin V, Gruntenko E. Inhibiting effect of cis-diamminodichloro platinum incorporated into liposomes on lymphogenic metastases of transplantable tumors in mice. *Dokl Acad Nauk SSSR* 1978, **242**, 473–476.
10. Yatvin M, Mühlensiepen H, Porschen W, Feinendegen L, Weinstein J. Selective delivery of liposome-associated cis-dichloro-diammine platinum (II) by heat and its influence on tumor drug uptake and growth. *Cancer Res* 1981, **41**, 1602–1607.
11. Gruntenko E, Nikolin V, Matienko N, Kaledin V, Vakrusheva T, Budker V. Liposomes as carriers of antineoplastic drugs in the treatment of neoplastic liver lesions. *Dokl Akad Nauk SSSR* 1982, **265**, 225–228.
12. Golla E, Ayres G. Spectrophotometric determination of platinum with O-phenylenediamine. *Talenta* 1973, **20**, 199–210.
13. Weber G, Robaye G, Delbrouck J, Roelandts I, Didberg O, Bartsch P, De Pauw M. Biomedical application of P.I.X.E. in University of Liège. *Nucl Instrum Meth* 1980, **168**, 551–556.
14. Raz A, Bucana C, Fogler W, Poste G, Fidler I. Biochemical, morphological and ultrastructural studies on the uptake of liposomes by murine macrophages. *Cancer Res* 1981, **41**, 487–494.
15. Lewis P, Knight D. In: Glauert A. *Practical Methods in Electron Microscopy*. Amsterdam, North-Holland, 1977, Vol 5, Part I, 112, 118, 119.
16. Heinen E. Mode d'action d'agents antimitotiques, en particulier d'agents liés à des macromolécules, sur des fibroblastes embryonnaires, des macrophages péritonéaux et des cellules tumorales d'Ehrlich. Thèse de Doctorat en Sciences biomédicales expérimentales. Université de Liège, 1977.
17. Gregoriadis G. In: Celis J, Graessmann A, Loyter A, eds. *Transfer of Cells Constituents into Eukariotic Cells*. New York, Plenum, 1980, 173–199.
18. Roberts J, Fraval H. Repair of *cis*-platinum (II) diammine dichloride-induced DNA damage and cell sensitivity. In: Prestayko A, Crooke S, eds. *Cisplatin Current Status and New Developments*. London, Academic Press, 1980, 57–78.
19. De Pauw-Gillet M, Heinen E, Houssier Cl, Fredericq E, Bassleer R. Effets du cis-chlorodiammineplatine (II) (cis-Pt II) sur l'ultrastructure de la chromatine de cellules

animales ou végétales et répercussions sur le cycle cellulaire. *C R Soc Biol* 1982, **176**, 364–368.
20. Sodhi A. Origin of giant cells in regressing sarcoma-180 after *cis*-dichlorodiammine platinum (II) treatment: a fine structural study. *J Clin Hematol Oncol* 1977, **7**, 569–579.
21. Daskal Y, Prestayko A, Crooke S. Morphological manifestations of cisplatin analogs in rats: an ultrastructural study. In: Prestayko A, Crooke S, eds. *Cisplatin Current Status and New Developments*. London, Academic Press, 1980, 249–269.
22. Houssier Cl, De Pauw-Gillet M, Hacha R, Fredericq E. Alterations in the nucleosome and chromatin structures upon interaction with platinum coordination complexes. *Biochim biophys Acta* 1983, **739**, 317–325.
23. Thompson L, Arquilla M, Simpkins H. The interaction of platinum complexes with nucleosomes investigated with fluorescent probes. *Biochim biophys Acta* 1982, **698**, 173–182.
24. Lippard, S. Binding of a platinum antitumor drug to its likely biological targets. *Inorg Chem Biol Med* 1980, **9**, 148–156.
25. Harrap K, Jones M, Wilkinson C, Clink H, Sparrow S, Mitchley B, Clarke S, Veasey A. Antitumor, toxic and biochemical properties of cisplatin and eight other platinum complexes. In: Prestayko A, Crooke S, eds. *Cisplatin Current Status and New Developments*. London, Academic Press, 1980, 193–212.
26. Behrens B, Yamamoto N, Brown R, Bryan G. Cytotoxicity of liposomes towards cultured human and rat bladder cancer cell lines. *Proc Am Assoc Cancer Res* 1979, **20**, 160.
27. Poste G, Papahadjopoulos D. Lipid vesicles as carriers for introducing materials into cultured cells: influence of composition on mechanisms of vesicles incorporation into cells. *Proc Natl Acad Sci USA* 1976, **73**, 1603–1607.
28. Pagano R, Weinstein J. Interactions of liposomes with mammalian cells. *Ann Rev Biophys Bioeng* 1978, **7**, 435–468.
29. Allen T, McAllister L, Mausolf S, Gyorffy E. A study of the interactions of liposomes containing entrapped anti-cancer drugs with the EMT6, S49 and $AE_1$ (transport deficient) cell lines. *Biochem biophys Acta* 1981, **643**, 346–362.
30. Lhoest-Gauthier M, De Pauw-Gillet M, Van Ophem Ph, Bassleer R. Effets de composés d'or libres ou incorporés dans des liposomes sur des cellules synoviales humaines en culture. *Arch Biol* 1984, **95**, 413–421.
31. Razi M, Otiro G, Sadler P. Ligang exchange reactions of gold drugs in model systems and in red cells. *Am Chem Soc* 1983, **19**, 371–384.