Preparation and cytotoxicity of cisplatin-containing liposomes

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Abstract

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We encapsulated cisplatin into stealth pH-sensitive liposomes and studied their stability, cytotoxicity and accumulation in a human small-cell lung carcinoma cell line (GLC4) and its resistant subline (GLC4/CDDP). Since reduced cellular drug accumulation has been shown to be the main mechanism responsible for resistance in the GLC4/CDDP subline, we evaluated the ability of this new delivery system to improve cellular uptake. The liposomes were composed of dioleoylphosphatidylethanolamine (DOPE), cholesteryl hemisuccinate (CHEMS), and distearoylphosphatidylethanolamine-polyethyleneglycol 2000 (DSPE-PEG$_{2000}$) and were characterized by determining the encapsulation percentage as a function of lipid concentration. Among the different formulations, DOPE/CHEMS/DSPE-PEG liposomes (lipid concentration equal to 40 mM) encapsulated cisplatin more efficiently than other concentrations of liposomes (about 20.0%, mean diameter of 174 nm). These liposomes presented good stability in mouse plasma which was obtained using a 0.24-M EDTA solution (70% cisplatin was retained inside the liposomes after 30 min of incubation). Concerning cytotoxic effects, they are more effective (1.34-fold) than free cisplatin for growth inhibition of the human lung cancer cell line A549. The study of cytotoxicity to GLC4 and GLC4/CDDP cell lines showed similar IC$_{50}$ values (approximately 1.4 μM), i.e., cisplatin-resistant cells were sensitive to this cisplatin formulation. Platinum accumulation in both sensitive and resistant cell lines followed the same pattern, i.e., approximately the same intracellular platinum concentration (4.0 x 10^{-17} mol/cell) yielded the same cytotoxic effect. These results indicate that long-circulating pH-sensitive liposomes, also termed as stealth pH-sensitive liposomes, may present a promising delivery system for cisplatin-based cancer treatment. This liposome system proved to be able to circumvent the cisplatin resistance, whereas it was not observed when using non-long-circulating liposomes composed of phosphatidylcholine, phosphatidylserine, and cholesterol.

Introduction

cis-Diamminedichloroplatinum (II) (CDDP) or cisplatin is one of the most effective chemotherapeutic agents used for the treatment of ovary, lung, testicle, head, and neck carcinoma (1-5). However, its use presents some inconveniences, such as development of resistance in human ovarian cell line A2780, cervical cancer cell line ME180 and colon carcinoma cell line LoVo; side effects, principally nephrotoxicity, and chemical incompatibility with other drugs used simultaneously in polychemotherapy, such as etoposide and thiotepa (6,7). The appearance of resistance during therapy is an important limitation to the use of CDDP, leading to treatment failure (8-11). The use of higher doses of CDDP is not desirable due to the toxicity of the drug to multiple organs, such as damage to the kidneys and bone marrow, intractable vomiting, peripheral neuropathy, deafness, seizures, and blindness (12-14). The reasons for resistance to cisplatin have been reported to be multifactorial. Four main events accompany it in most cases: i) decreased accumulation of drug concentration (to below that necessary for cytotoxic activity), ii) increased levels of sulfur-containing molecules such as glutathione or metallothionein (which could play a role in metal detoxification), iii) enhanced repair of DNA damage caused by CDDP-DNA adducts by nucleotide excision repair proteins such as ERCC1, XPA, and XPB which can remove DNA adducts produced by CDDP, and iv) increased tolerance to CDDP-DNA adducts as a consequence of deficiencies in the mismatch repair system and enhanced replication bypass (15-18). Among the various resistance mechanisms involved, decreased cellular accumulation of CDDP has been demonstrated in most cases (19-24). Reduced intracellular CDDP concentration results in decreased DNA platination, which leads to CDDP resistance. The mechanism by which CDDP enters cells is not known. It has been postulated that cellular uptake of CDDP occurs by passive diffusion; however, some reports support a protein-mediated transport (21,24-26).

Thus, the employment of new delivery systems of CDDP such as stealth pH-sensitive liposomes can be a strategy to overcome these limitations. The encapsulation of CDDP into pH-sensitive liposomes can improve its entry into cells, can lead to an another pharmacokinetics profile, reducing or eliminating the side effects, and can allow the association with other drugs without the appearance of degradation products. These liposomes are generally taken up by the cells through an endocytosis process. The release of CDDP into the cytoplasm results from the ability of these liposomes to cross the endosomal membrane. Inside endosomes, the stealth pH-sensitive liposomes are exposed to an acidic medium, pH 5.5-6.5, which provokes their fusion with and the destabilization of the endosome membrane. Then, the encapsulated content is delivered into the cytoplasm (27,28). Therefore, in the present study, we investigated the encapsulation of CDDP into stealth pH-sensitive liposomes, the cytotoxic activity of the preparation, and its ability to circumvent CDDP resistance using A549, A431, BHK-21, and GLC4, GLC4/CDDP cell lines, respectively.

Material and Methods

Material

Cisplatin was supplied by Quiral Química do Brasil S.A. (Juiz de Fora, MG, Brazil). Distearoylphosphatidylethanolamine-polyethyleneglycol (DSPE-PEG) and dioleoylphosphatidylethanolamine (DOPE) were supplied by Lipoid GmBh (Ludwigshafen, Germany). Cholesteryl hemisuccinate (CHEMS) was purchased from Sigma (St. Louis, MO, USA), and sodium chloride was purchased from Merck (Rio de Janeiro, RJ, Brazil).

Preparation of liposomes

Initially, DOPE, CHEMS, and DSPE-PEG$_{2000}$ were dissolved in chloroform (lipid concentration 20, 30, and 40 mM; molar ratio 5.7:3.8:0.5) and were transferred to a round bottom flask and submitted to evaporation. The lipid film obtained was dissolved in ethyl ether and then added to the 2 mg/mL CDDP solution prepared in 0.9% NaCl. The ratio of the aqueous to ether phase was 1:3. The resulting mixture was submitted to fast vortex agitation to produce a type of water/oil emulsion. Next, ethyl ether was evaporated, with the resulting formation of liposomes. These liposomes were submitted or not to filtration through 0.4-μm and 0.2-μm polycarbonate membranes (5 cycles each) and the unencapsulated CDDP was eliminated by ultracentrifugation with a Sorvall Ultra 80 ultracentrifuge (Albertville, MN, USA) at 150,000 g at 10ºC for 60 min.

Characterization of liposomes

The liposomes were characterized on the basis of their encapsulation percentage, size and zeta potential. The encapsulation percentage of CDDP into liposomes was determined by high-performance liquid chromatography. The chromatographic apparatus consisted of a Model 515 pump (Waters Instruments, Milford, MA, USA), a Model 717 Plus auto-injector (Waters Instruments) and a Model 2487 variable wavelength UV detector (Waters Instruments) connected to the Millennium software. Separations were done using Lichrospher® 100 NH2 guard column, 4 x 4 mm, 5 μm (Merck, Darmstadt, Germany) connected to a 25 cm x 4 mm Lichrospher® 100 NH2 column, 10-μm particles (Merck). The eluent system was composed of methanol/ethyl acetate/N,N-dimethylformamide/water, 4:4:1:1, and the flow rate was 1.0 mL/min. Samples (20 μL) were injected and the eluate absorbance was monitored at 310 nm. The amount of CDDP was

determined in the stock solution employed for the preparation of liposomes and in the supernatant. The encapsulation percentage (EP) was calculated as:

$$EP = \frac{[CDDP]_{\text{liposomes before ultracentrifugation}} \text{ minus } [CDDP]_{\text{supernatant}}}{[CDDP]_{\text{liposomes before ultracentrifugation}}} \times 100$$

The loading capacity was assessed by measuring inorganic phosphorus (29). The mean diameter of the liposomes-containing CDDP was determined by the quasi-elastic light scattering, at 25ºC and at an angle of 90º. The zeta potential was evaluated by the determination of electrophoretic mobility at the 90º angle. The measurements were performed in triplicate using the 3000 HS Zetasizer equipment (Malvern Instruments, Worcestershire, England). The samples were diluted with a 0.9% NaCl.

Liposome stability

Stealth pH-sensitive liposomes containing CDDP were diluted 10-fold in 0.9% NaCl, pH 6.5, or mouse plasma, pH 7.4, and incubated at 37ºC for 30 min. The platinum released from liposomes containing CDDP was separated and determined by ultracentrifugation at 150,000 g, 10ºC, for 60 min followed by graphite furnace atomic absorption spectrometry (Springvale, Victoria, Australia).

Cytotoxicity assay

The human lung cancer cell line A549 and human cervix squamous carcinoma cell line A431 were cultured in modified DEMEN medium supplemented with 10% fetal bovine serum. The Baby Hamster Kidney cell line (BHK-21) was grown in RPMI 1640 medium supplemented with 10% fetal bovine serum. All cell lines were maintained at 36.5ºC in a humidified 2.5% CO$_2$ atmosphere. An aliquot containing 4 x 10^3 BHK

cells, 5×10^3 A549 or A431 cells was distributed in 96-well plates. After 24 h of growth, the cells were submitted to treatment with free CDDP, stealth pH-sensitive liposomes containing CDDP and empty stealth pH-sensitive liposomes. The CDDP concentration employed was 79.4 nM, 3.7 and 0.46 μM for the A431, A549 and BHK cells, respectively. These concentrations correspond to the IC_{50} of CDDP for each cell line. The treatment was performed on three consecutive days, with a change of the culture medium for each new treatment. Seventy-two hours after the initial treatment, cell viability was assessed by 3-[4,5-dimethyl-thiazol-2yl]-diphenyltetrazolium bromide (MTT) reduction. First, 10 μL of a 5-mg/mL tetrazolium salt solution was added to each well of the plate. After 4 h, MTT crystals were solubilized in 150 μL dimethylsulfoxide. Absorbance readings were performed at 600 nm using a Stat Fax 2100 spectrophotometer (Awareness Technology, Inc., Palm City, FL, USA). Data were analyzed by two-way ANOVA and the means were compared by applying the Bonferroni test ($P \leq 0.05$) using the GraphPad Prism, version 4.0, software.

Evaluation of resistance of cisplatin

The GLC4 cell line was derived from the pleural effusion of a patient with small cell lung carcinoma in the laboratories of Prof. E.G.E. de Vries (Department of Internal Medicine, University Hospital, Groningen, The Netherlands). The GLC4/CDDP subline was obtained by continuous exposure to CDDP. The cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum at 37ºC in a humidified 5% CO_2 atmosphere. Cultures were initiated at 10^5 cells/mL and grew exponentially to about 10^6 cells/mL within three days. Cell viability was checked by Trypan blue exclusion. The number of cells was determined by Coulter counter analysis. For the long-term growth

inhibition 1×10^5 cells/mL were cultured for 72 h in the presence and absence of different concentrations of CDDP encapsulated or not into stealth pH-sensitive liposomes. The sensitivity to the drug was evaluated by the IC_{50}, i.e., the drug concentration needed to inhibit cell growth by 50%. A resistance factor (RF) was obtained by dividing the IC_{50} of resistant cells by the IC_{50} of sensitive cells. RF is equal to 6.3 for CDDP and CDDP resistance is stable for at least 8 months (24).

Determination of cellular platinum concentrations

Cells were incubated with different concentrations of CDDP or CDDP containing stealth pH-sensitive liposomes for 3 days. After incubation, an aliquot was taken and washed twice with ice-cold PBS and the pellet was resuspended in 65% HNO_3. Platinum concentration was determined by atomic absorption spectroscopy using a Varian model Zeeman 220 spectrophotometer (Mulgrave, Victoria, Australia) equipped with a graphite tube atomizer and an autosampler.

Results and Discussion

Preparation of stealth pH-sensitive liposomes containing CDDP

Formulation studies showed that the encapsulation of CDDP was significantly improved by increasing the amount of lipids used. Percent CDDP entrapment ranged from 6.6 to 18.6% depending on the lipid concentration (Table 1). The loading capacity of liposomes also increased when the lipid concentration varied only from 30 to 40 mM (Table 1). However, these liposomes showed a heterogeneous size distribution (444.5 nm, polydispersity index equal to 0.6) that could induce the destabilization of the vesicles, provoking the release of CDDP. Thus, these liposomes were submitted to extrusion

against 0.4 and 0.2 μm polycarbonate membranes before purification by ultracentrifugation. However, the size reduction did not alter the entrapment percentage (20.0%), but produced a vesicle suspension of homogeneous diameter (174 nm, polydispersity index equal to 0.03) and a zeta potential equal to 1.6 mV. The liposomes containing the largest amount of CDDP were chosen for the evaluation of cytotoxicity and resistance.

Liposome stability

Stealth pH-sensitive liposomes containing CDDP showed good stability in protein-rich medium such as mouse plasma. Approximately 70% CDDP remained inside the liposomes after their incubation in this medium. The leakage of CDDP from the liposomes exposed to 0.9% NaCl was similar, with no significant difference ($P < 0.05$). Probably, the release of CDDP from liposomes was due to heating (37ºC) and not to the destabilization provoked by interactions with plasma proteins.

Cytotoxicity

Figure 1 illustrates the cytotoxic effects of CDDP encapsulated or not into stealth pH-sensitive liposomes on normal and tumor cell lines. The cytotoxic activity of stealth pH-sensitive liposomes containing CDDP was higher than that of free CDDP against the A549 cell line ($P < 0.05$). However, these liposomes did not prove to be more effective against the A431 cell line. Cell growth inhibition was lower with the encapsulation of CDDP into stealth pH-sensitive liposomes. The same effect was observed for the GLC4 cell line (Table 2). The cellular sensitivity to CDDP encapsulated into stealth pH-sensitive liposomes was lower than for free CDDP, as revealed by the determination of the IC_{50}. These findings suggest that the cytotoxic activity of these liposomes containing CDDP is dependent on the cell line type. Statistical analysis indicated the influence of cell type on the response to treatment with free CDDP or with stealth pH-sensitive liposomes containing CDDP. The A549 cells showed higher sensitivity to treatment with stealth pH-sensitive liposomes containing CDDP than A431 cells ($P = 0.0006$). Perhaps, the cell line-associated differences in the efficacy of stealth pH-sensitive liposomes containing CDDP are related to the uptake mechanisms

Table 1. Effect of lipid concentration on the encapsulation of CDDP into stealth pH-sensitive liposomes.

Lipid concentration during preparation of liposomes	Entrapment (%)	Loading capacity (mg CDDP/nmol lipid)
20 mM	6.95 ± 0.32	10.96 ± 0.35
30 mM	8.80 ± 0.29	10.20 ± 0.92
40 mM	18.60 ± 1.26	14.32 ± 0.81

Data are reported as means ± SD for three determinations. CDDP = cis-diamminedichloroplatinum (II) (cisplatin).
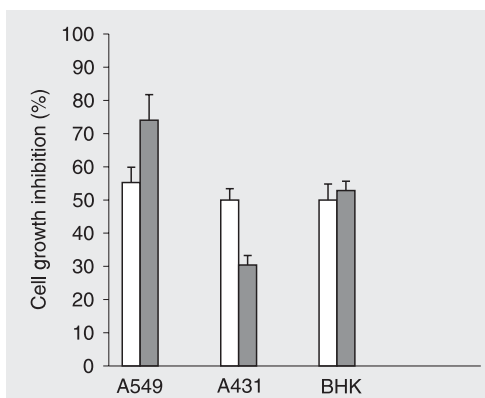

Figure 1. Cytotoxic activity of free CDDP (open bars) and of stealth pH-sensitive liposomes containing CDDP (filled bars). Data are reported as means ± SD and were obtained from six experiments. CDDP = cis-diamminedichloroplatinum (II) (cisplatin); A549 = human lung cancer cell line; A431 = human cervix squamous carcinoma cell line; BHK-21 = Baby Hamster Kidney cell line.

Table 2. IC_{50}, resistance factor (RF) and intracellular platinum concentrations after 3 days of incubation with CDDP or liposomal CDDP.

Compound	Cell line	IC_{50} (μM)	RF	$[Pt]_i$ x 10^{17} (mol/cell)
CDDP	GLC4	0.40 ± 0.05		9.8
CDDP	GLC4/CDDP	2.50 ± 0.20	6.3	10.7
Liposomal CDDP	GLC4	1.32 ± 0.10		4.0
Liposomal CDDP	GLC4/CDDP	1.45 ± 0.15	1.1	4.0

IC_{50} is the CDDP concentration required to inhibit cell growth by 50%. The resistance factor (RF) is the IC_{50} of the resistant cells divided by the IC_{50} of the sensitive cells. $[Pt]_i$ is the intracellular platinum concentration. Data are reported as the mean ± SD of triplicate determinations. CDDP = cis-diamminedichloroplatinum (II) (cisplatin).

involved in each cell line. Lim et al. (30) demonstrated different levels of liposomal mitoxantrone accumulation in LS180 human colon carcinoma tumors compared to
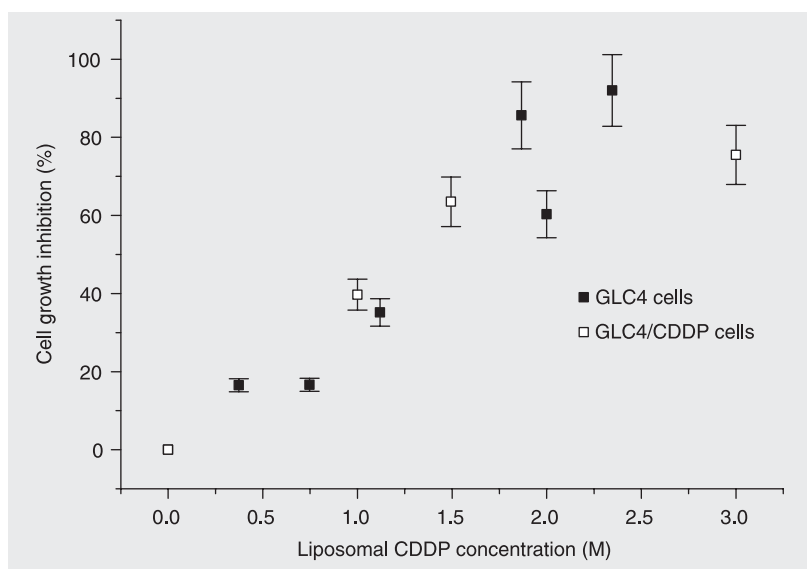

Figure 2. Dose-response curves of GLC4 and GLC4/CDDP cells for stealth pH-sensitive liposomes of CDDP obtained after 3 days of incubation. Data are reported as means ± SD for three experiments. GLC4 = human small-cell lung carcinoma cell line; CDDP = cis-diamminedichloroplatinum (II) (cisplatin).
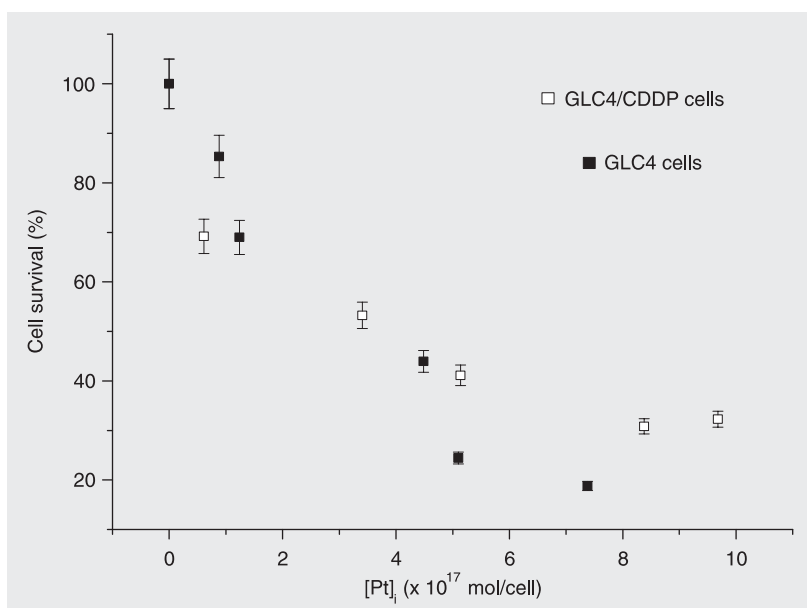

Figure 3. Cell survival as a function of intracellular platinum concentration after equitoxic drug exposure for 3 days. Data are reported as means ± SD for three independent experiments. GLC4 = human small-cell lung carcinoma cell line; CDDP = cis-diamminedichloroplatinum (II) (cisplatin).

A431 tumors. This finding has been attributed to the more rapid liposome uptake in the LS180 cell line. The cytotoxicity of free or liposome-encapsulated CDDP on the BHK cell line was identical. This cell line was chosen for the assay due to the renal toxicity induced by the use of CDDP. In spite of this finding, it should be pointed out that the biodistribution behavior of the free drug and of the drug encapsulated into nanostructured systems is not the same. Júnior et al. (31) and Newman et al. (32) obtained different pharmacokinetics profiles from administration of liposomal formulations containing CDDP in comparison with free CDDP. In both studies, a lower renal accumulation of CDDP from liposomal CDDP treatment was observed than that from free CDDP. Thus, the *in vivo* toxicologic response to stealth pH-sensitive liposomes containing CDDP can be modified, avoiding the occurrence of kidney failure. No cell growth inhibition was observed with empty stealth pH-sensitive liposomes.

Drug sensitivity

The effect of stealth pH-sensitive liposomes containing CDDP on the growth of the GLC4 and GLC4/CDDP cell lines is shown in Figure 2. One can observe that the response of both cell lines to a given dose of this new cisplatin formulation was comparable. Table 2 shows the drug concentration that inhibits cell growth by 50% and the RF obtained. For the sake of comparison, CDDP data are also shown. Intriguingly, the cytotoxic activity of stealth pH-sensitive liposomes containing CDDP on the sensitive cell line was inferior to that of free CDDP. This fact is probably related to different uptake mechanisms of the free and the encapsulated CDDP.

Drug accumulation after equitoxic exposure

The percentage of growth inhibition ver-

sus the content of platinum within the cell determined in GLC4 and in GLC4/CDDP is plotted in Figure 3. Comparing platinum accumulation in the two cell lines, one notices that almost the same levels of platinum are necessary to produce a certain cytotoxic effect. For example, incubation of CDDP with stealth pH-sensitive liposomes at IC_{50} doses yielded a comparable $[Pt]_i$ for both sensitive and resistant cells of about 4 x 10^{-17} mol/cell. We have previously shown that the accumulation of CDDP in both GLC4 and GLC4/CDDP cell lines follows the same pattern, i.e., incubation with equitoxic doses yields practically the same intracellular platinum levels. For example, incubation with CDDP at IC_{50} doses (glc4 = 0.4 μmol/L and GLC4/CDDP = 2.5 μmol/L) yielded comparable $[Pt]_i$ of 10 x 10^{-17} mol/cell for both sensitive and resistant cells. These results show the importance of transport as a mechanism of resistance to CDDP exhibited by GLC4/CDDP cells. In the case of free CDDP, it was necessary to use a higher dose to force its uptake by GLC4/CDDP cells, a fact that was not observed with the use of stealth pH-sensitive liposomes containing CDDP. CDDP encapsulated into stealth pH-sensitive liposomes enters sensitive cells as easily as resistant cells. The liposomal formulation exhibited the same efficacy against CDDP-sensitive and CDDP-resistant cells, with an RF equal to 1.1 (Table 2). This factor is much lower than that for non-encapsulated CDDP, which is equal to 6.3 (24). Thus, these liposomes were able to overcome the resistance mechanisms. In addition, the lower intracellular platinum concentration detected when the cells were incubated with an equitoxic CDDP concentration (IC_{50}) suggests the occurrence of a different transport mechanism for stealth pH-sensitive liposomes. Aqueous formulations of CDDP are solutions of the native and hydrated forms of the drug in equilibrium, which is dependent on the pH and Cl^- concentrations. Chloride ions can be replaced with water molecules,

which in turn can be hydrolyzed, yielding hydroxy species. The most important product, the monohydrated species, $[Pt(NH_3)_2(OH_2)Cl]^+$, is formed by the following reaction:

$$cis\text{-}[Pt(NH_3)_2Cl_2] + H_2O \leftrightarrows cis\text{-}[Pt(NH_3)_2(OH_2)Cl]^+ + Cl^-$$
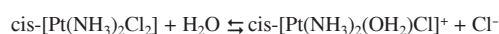
We have previously proposed that CDDP uptake by sensitive GLC4 cells is the sum of two components: an energy-dependent pathway and passive diffusion. The active component is deficient in the GLC4/CDDP subline. The actively transported species is hydro-derivative, probably $[Pt(NH_3)_2(H_2O)OH]^+$ or $[Pt(NH_3)_2(H_2O)Cl]^+$ (24). Cationic aquated species are more reactive with DNA, producing intra- and inter-strand adducts and, consequently, the cytotoxic effects. Therefore, one can speculate that the CDDP delivery by stealth pH-sensitive liposomes favors the formation of hydrated species. It is known that CDDP is almost completely converted to the monohydro species at pH 6.0 (80%) (33). As the stealth pH-sensitive liposomes enter the cells by endocytosis, the release of CDDP might occur as a consequence of the acidification process inside the endosomes, as explained above. Inside late endosomes the pH ranges from 5.5 to 6.0, and consequently, the main species present is the aquohydroxy-derivative. This could explain the achievement of a similar cytotoxicity level with the presence of a lower intracellular platinum concentration.

Finally, it is noteworthy that the stealth pH-sensitive liposomes might offer advantages over conventional liposomes due to their ability to deliver their contents into the cytoplasm. These liposomes can be an alternative to solve the clinical failure observed with the use of stealth liposomes in the treatment of neck and head carcinoma (34). The authors believe that the clinical inefficacy results from the low release of CDDP. To solve this problem would be appropriate to improve liposome composition. Thus, the

present study allowed us to determine more adequate conditions for the encapsulation of CDDP into stealth pH-sensitive liposomes. These stealth pH-sensitive liposomes are stable in plasma and this finding is important to the *in vivo* evaluation of their antitumoral efficacy and pharmacokinetics. Moreover, these liposomes proved to be active against a type of lung tumor cell line (A549) and to provide an excellent strategy to circumvent the pre-clinical resistance to treatment with CDDP. These carriers were able to introduce the same level of CDDP into resistant and sensitive cells after incubation with equal CDDP concentrations.

Acknowledgments

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