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RESEARCH ARTICLE

Effect of cisplatin containing liposomes formulated by unsaturated chain-containing lipids on gynecological tumor cells

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Abstract

Gynecological tumors are major therapeutic areas of platinum-based anticancer drugs. Here, we report the characterization and *in vitro* biological assays of cisplatin-containing Egg L- α -phosphatidylcholine liposomes with different amounts of cholesterol. Dynamic light scattering estimated sizes of all obtained liposomes in the 100 nm range that are suitable for *in vivo* use. On the basis of these data and of the drug loading values, the best formulation has been selected. Stability and drug release properties of platinum-containing liposomes have been verified in serum. The growth inhibitory effects of both liposomal and free drug in a panel of ovarian and breast human cancer cell lines, characterized by a different drug sensitivity, give comparable or better results with respect to free cisplatin drug.

Keywords

CDDP, cisplatin, drug delivery, gynecological tumors, liposomes

History

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Introduction

Since the clinical introduction, more than 40 years ago, platinum containing drugs are widely employed as chemotherapeutic agents for the treatment of a variety of human cancers (Kelland, 2007). Ovarian and breast cancer are major therapeutic areas of platinum-based anticancer drugs. Cisplatin (CDDP) is very effective in the treatment of ovarian cancer, and, recently, the interest in platinum-based chemotherapy for breast cancer has also been renewed, based on the hypothesis of greater susceptibility of triple-negative and *BRCA1/2*-mutant tumors (Muggia & Safra, 2014).

Although very active, the dose limiting side effects of CDDP, including nephrotoxicity, peripheral neuropathy and ototoxicity, hamper dose increase and inhibit its extensive clinical use (Burger et al., 1999; Howe-Grant & Lippard, 1980; Reedijk, 1999). Moreover, in many cases, patients respond to first-line treatment, but the majority ultimately develops a platinum-resistant disease. Several mechanisms underlying the multifactorial CDDP-resistance phenotype have been described (Galluzzi et al., 2012). Importantly, a decreased intracellular drug accumulation is a common feature of CDDP-resistant tumor cell lines; and a relationship between tumor tissue platinum concentration and therapeutic response in clinical settings has also been recently highlighted

(Kim et al., 2012). These findings demonstrate that mechanisms underlying platinum drug transport and accumulation in tumor cells are important determinants for tumor selectivity and treatment effects.

Many ways have been searched to overcome these drawbacks. One opportunity is to develop new platinum analogs or platinum prodrugs, which are able to decrease the cellular proliferation and at the same time reduce the side effects (Lovejoy & Lippard, 2009). However, some drawbacks and resistance persist and neither of analogs showed significantly better results than CDDP (Wheate et al., 2010).

Another way is based on nanotechnologies that are recently developed. Nanocarrier-based delivery of platinum drugs to the tumor sites is a promising strategy for improving tumor selectivity, and encouraging preclinical and clinical results have been reported (Oberoi et al., 2013).

To the best of our knowledge, five platinum containing supramolecular aggregates are in clinical evaluation. The first liposomal formulation studied in the clinic for the delivery of a CDDP analog (cis-bis-neodecanoato-trans-R, R-1,2-diaminocyclohexane platinum II, NDDP) was L-NDDP (AroplatinTM, Antigenics Inc, Lexington, MA) (Vadiei et al., 1992). Liposomes were obtained mixing 1,2-dimyristoylphosphatidylcholine (DMPC) and 1,2-dimyristoylphosphatidylglycerol (DMPG) lipids. Preclinical data showed that L-NDDP had a completely different biodistribution from that of NDDP, with an accumulation of platinum in major organs, such as the liver, spleen and lymph nodes.

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Another long-circulating aggregate is SPI 077 (Alza Corporation, Mountain View, CA). This drug is based on liposomes formulated by Hydrogenated Soy Phosphatidylcholine (HSPC)/cholesterol/1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[amino(polyethylene glycol)-2000] (DSPE-PEG2000) and loaded with CDDP by using the loading passive method (Newman et al., 1999). Although this supramolecular aggregate shows enhanced pharmacokinetic properties with respect to CDDP, SPI 077 did not produce significant clinical response rates in several Phase II studies. The major limit of lack of therapeutic efficacy is due to slow and low release of platinum drug from aggregates, as shown in preclinical studies (Harrington et al., 2001).

A more recent CDDP-containing long-circulating liposomal formulation is LipoplatinTM (Regulon Inc, Mountain View, CA) (Stathopoulos & Boulikas, 2012). This formulation differs from the SPI 077 in several basic principles including loading method, based on reverse micelles, anionic lipids like 1,2 dipalmitoylphosphatidylglycerol (DPPG) added in the phospholipids mixture and a higher drug content 1:10 versus 1:70 drug/lipid reported for SPI 077.

In several Phase I, II and III studies, Lipoplatin was shown to reduce renal toxicity, peripheral neuropathy, ototoxicity, and myelotoxicity substantially with improved or comparable efficacy to CDDP (Boulikas, 2009). The same company developed LipoxalTM (Stathopoulos et al., 2006), an oxaliplatin delivering liposomal formulation. In a Phase I study using Lipoxal, reduction respect to free oxaliplatin of myelotoxicity, nausea and peripheral neuropathy was observed. Further clinical tests will be needed to demonstrate the improvement of antitumor activity of Lipoxal over free oxaliplatin.

LiPlaCis (LiPlasome Pharma ApS – Denmark) (de Jonge et al., 2010), liposomes obtained mixing 1,2-distearoylphosphatidylcholine (DSPC)/1,2-distearoylphosphatidylglycerol (DSPG)/DSPE-PEG2000 were designed to be degraded by secretory phospholipase A₂ (PLA₂), a relatively tumor selective enzyme, releasing the encapsulated CDDP. Renal toxicity and acute infusion in the Phase I stage of LiPlaCis led to early cessation of this particular formulation: LiPlaCis requires reformulation to enable further development. In parallel, the same company is testing Liploxa, another oxaliplatin-loaded liposomal formulation. Many different other supramolecular aggregates have been developed in the last years, in preclinical phase, using a novel approach based on hybrid nucleoside-lipids (Khiati et al., 2011). The nucleoside polar heads guide the self-assembly of the aggregates into highly loaded and stable CDDP nanoparticles.

Very innovate systems based on aggregation of platinum containing monomers were recently reported for delivery of platinum-based drugs. These systems integrate drugs into the lipid composition, leading to an extremely high drug-to-lipid ratio. In Aryal et al. (2012), two hydrophobic acyl chains were bound to the Pt(II) center to provide amphiphilic properties to the platinum monomer; in Accardo et al. (2013), the same results were achieved coordinating the metal to amino-EtGly-PEG₂₇-(C18)₂ amphiphilic. Both molecules are able to self-assemble in supramolecular aggregates, and in the second case the platinum-based monomer is also able to

co-aggregate with a second amphiphilic monomer containing the octreotide bioactive peptide able to recognize somatostatin receptor type II (SSTR2) overexpressed in many cancer tissues. However, these studies provide only preliminary results without indication on their *in vivo* effects.

The literature suggests that design and preparation of liposomal nanovehicles require to achieve a balance between their stability *in vivo*, encapsulation efficiency, drug release, biodistribution and cytotoxic activity. The current liposomal formulations have primarily taken advantage of reduced systemic toxicity rather than increased efficacy.

In this study, we encapsulate CDDP in Egg L- α -phosphatidylcholine (PC) liposomes containing different amounts of cholesterol. The Egg PC is a mixture of molecular species of PC differing in fatty acyl chains, and it includes a considerable amount of unsaturated fatty acids, such as arachidonic (C20:4) and decasohexanoic (C22:6) acids. Its stability and properties are well known in the literature (Grit et al., 1993).

Phosphatidylcholine was selected as a phospholipid, since it represents a good compromise between saturated or unsaturated phospholipids. The unsaturated bond increases fluidity and permeability with respect to HSPC used in other preparations and currently tested in clinical phase. The lower stability of the aggregates may be increased adding cholesterol to the phospholipids mixture. The presence of cholesterol exerts a deep influence on the properties of lipid bilayers of the liposomes. It has been well known that cholesterol addition to unsaturated lipids decreases both membrane permeability to water and flexibility of the surrounding lipid chains, favoring retention of encapsulated content.

The SPI 077 formulation, containing 44% of cholesterol in weight, shows a low release of platinum drug from aggregates (Harrington et al., 2001). Therefore, we prepared two different formulations using lower amounts of cholesterol (PC/Chol weight ratio 80:20 and 70:30) in order to increase platinum release. The growth inhibitory effects of both liposomal drug and free drug in a panel of ovarian and breast human cancer cell lines characterized by different drug sensitivity is investigated.

Materials and methods

Materials

Phosphatidylcholine and cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabaster, AL). SnakeSkinTM Dialysis Tubing was purchased by Thermo Scientific Pierce Protein Research Products (Waltham, MA). CDDP and all other chemicals were commercially provided by Sigma-Aldrich (Bucks, Switzerland) or LabScan (Stillorgan, Dublin, Ireland) and were used as received unless further purification. All the solutions were prepared by weight with bidistilled water.

Liposomes were extruded using a thermobarrel extruder system (Northern Lipids Inc, Vancouver, BC, Canada). Inductively coupled plasma atomic emission (ICP-AES) measurements were carried out using an inductively coupled plasma atomic emission spectrometer (ICP SPECTRO Arcos with EndOnPlasma torch; Spectro Analytical, Kleve, Germany) equipped with a capillary cross-flow nebulizer.

Liposomes preparation and CDDP loading

Empty aggregates composed of PC and PC/Chol at different molar ratios were prepared by the thin film method. The two amphiphiles dissolved in a small amount of MeOH/CHCl₃ (50/50) were mixed in appropriate amounts to obtain a final lipid concentration of 2 mM. Subsequently, a thin film was obtained by evaporating the organic solvents by slowly rotating the vial containing the solution under a gentle stream of nitrogen. Lipid films were then hydrated in 100 mM phosphate-buffered saline (PBS) containing 0.9% w/w of NaCl, sonicated for 30 min and extruded 10 times at room temperature through a polycarbonate membrane with 100 nm pore size, using a thermobarrel extruder system under N₂.

CDDP was encapsulated into liposomes using the passive equilibration loading method. Preformed liposomes were warmed at 60 °C for 5 min before adding to pre-warmed CDDP powder. The external CDDP concentration used was 1 mg/1 mL. The mixture was incubated at the above indicated temperature for 1.5 h in a water bath under stirring, then cooled to room temperature. Unencapsulated CDDP was removed first by centrifugation at 13 400 rpm at room temperature for 15 min and then loading the supernatant on pre-equilibrated Sephadex G50 column. Liposomal fractions were collected and analyzed by ICP-MS to determine platinum content.

Dynamic light scattering characterization

Dynamic light scattering (DLS) measurements were carried out using a Zetasizer Nano ZS (Malvern Instruments, Westborough, MA) that employs a 173° backscatter detector. Other instrumental settings are measurement position (mm): 4.65; attenuator: 8; temperature 25 °C; cell: disposable sizing cuvette. Before starting with DLS measurements, previously prepared samples were diluted at final concentration of 2.0×10^{-4} M and centrifuged at room temperature at 13 000 rpm for 5 min. For each formulation, RH and P.I. were calculated as the mean of three measurements on three different batches.

Serum stability

Cisplatin-loaded liposomes were prepared in 100 mM phosphate buffer at pH 7.4 containing 0.9% NaCl at 4 mM lipid concentration and then diluted at 2 mM with fetal bovine serum (FBS; Biowest, Nuaille, France). The colloidal suspension was stirred at 37 °C for 72 h. Samples withdrawn at different time points (30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h, 36 h, 48 h and 72 h) were diluted with PBS (1:50 v/v) and analyzed by dynamic light scattering as described earlier.

CDDP release in serum

A total of 500 μ L of FBS were added to 500 μ L of a 4 mM CDDP-loaded liposomes prepared according the protocol described above. The final FBS/liposome solution (50/50 v/v) was transferred into a dialysis bag (MW cut-off = 3500 Da). Then the sample was placed into 15 mL of FBS/PBS (50/50) mixture and incubated under stirring for 24 h at 37 °C. At appropriate intervals, 2 mL aliquots of the dialyzed serum

solution were withdrawn and immediately replaced with an equivalent amount of fresh serum. The extent of CDDP release was evaluated by ICP-AES analysis as percentage of the ratio between the amount of released metal and of the total metal previously loaded into the liposomes.

A CDDP standard solution (1000 ppm) was used to prepare nine solutions by sequential dilution in 5% (v/v) aqua regia (HCl/HNO₃ 3:1 v/v), to yield the calibration curves in the range 2–150 ppb. Each sample was mineralized with aqua regia, using nitric acid and hydrochloric acid in bidistilled water, to a final aqua regia content of 5% v/v. Analytical determinations were performed using a plasma power of 1.4 kW, a radiofrequency generator of 27.12 MHz and an argon gas flow with nebulizer, auxiliary and coolant set at 0.8, 0.8 and 13 L min⁻¹, respectively.

Tumor cell lines and *in vitro* cell growth inhibition assay

Tumor cell lines representative of breast (MCF7, MDA-MB-231) and ovarian (SKOV-3) cancers were obtained from the National Cancer Institute, Biological Testing Branch (Frederick, MD), and maintained in the logarithmic phase at 37 °C in a 5% CO₂ humidified air in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin and streptomycin (100 U/mL and 0.1 mg/mL, respectively). The human ovarian cancer cell line A2780 from an untreated patient, and the A2780cp8 CDDP-resistant subline were originally obtained from Dr R Ozols (Behrens et al., 1987) and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 μ M glutamine, 10 mg/mL gentamycin and 10 μ g/mL insulin.

The growth inhibitory effect of compounds under investigation was evaluated by using the sulforhodamine B assay (Skehan et al., 1990). Briefly, cells were seeded into 96-well microtiter plates in 100 μ L at a plating density of 5000 cells/well. After seeding, microtiter plates were incubated at 37 °C, 5% CO₂, 95% air and 100% relative humidity for 24 h prior to addition of the compounds. After 24 h, several samples of each cell line were fixed *in situ* by a gentle addition of cold 50% (w/v) trichloroacetic acid (TCA, 10% final concentration), to represent a measurement of the cell population at the time of compound addition. Cells were treated for 72 h with different concentrations of free CDDP, liposomal CDDP, or empty liposome dissolved in 0.1 M PBS, pH 7.4, 0.9% NaCl and stepwise diluted to the desired final concentration with complete cell culture medium. At the end of treatment time, the cells were fixed *in situ* with cold TCA and incubated for 1 h at 4 °C. The supernatant was discarded, and the plates were washed four times with tap water and air-dried. Sulforhodamine B (SRB) solution (100 μ L) at 0.4% (w/v) in 1% acetic acid was added to each well, and plates were incubated for 30 min at room temperature. After staining, unbound dye was removed by washing five times with 1% acetic acid and the plates were air-dried. Bound stain was then solubilized with 10 mM Trizma base and the absorbance was read on an automatic plate reader at 515 nm. The compound concentration able to inhibit cell growth by 50% (IC₅₀ \pm SD) was then calculated from semilogarithmic dose-response plots.

Results and discussion

Preparation and characterization of CDDP-containing liposomes

Three different lipid formulations based on EggPC were prepared, with 0, 20 and 30% (mol/mol) of Chol. All liposomes were prepared starting from a lipid film, obtained in MeOH/CHCl₃ (50/50) mixture, hydrated with 0.9% w/w of NaCl containing PBS buffer, by extensive bath sonication. The colloid solutions were extruded at room temperature through a polycarbonate membrane with 100 nm pore size in order to reduce liposome size and obtain unilamellar vesicles.

Cisplatin was loaded into liposomes using the passive equilibration loading method (Woo et al., 2008). The unencapsulated CDDP was removed first by centrifugation and then eluting the supernatant on pre-equilibrated Sephadex G50 column (GE Healthcare Europe, Freiburg, Germany). The drug loading contents (DL), measured with respect to the PC phospholipid content, and the encapsulation efficiencies are reported in Table 1. The encapsulation CDDP amount was quantified by means of ICP-AES on liposomal fraction.

A progressive increase of encapsulated CDDP was observed starting from 0 to 30% of cholesterol. As it concerns, CDDP encapsulation and release from liposomes, several factors appear to be critical, and in general factors improving loading could reduce retention and vice-versa (Newman et al., 1999; Stathopoulos & Boulikas, 2012). Most of studied CDDP-containing liposomes are based on formulations having high cholesterol percentages in order to stabilize the liposome membrane. However, it is known that these formulations present a difficult CDDP release *in vivo*. In our study, we used an unsaturated phospholipid to increase membrane fluidity coupled with increasing amounts of cholesterol. A formulation with 20% of cholesterol seems a good compromise to allow a sufficient CDDP loading, leaving a fluid membrane to permit an efficient drug release *in vivo*.

The CDDP-loaded liposomes were characterized by DLS technique. The data are reported in Table 1. Measurements were performed at $\theta = 173^\circ$ at a concentration of 5×10^{-4} mol·kg⁻¹ in 2.0×10^{-4} M PBS buffer solution. All aggregates show a monomodal distribution indicating the presence of a single population of aggregates. At infinite dilution, R_H values can be reasonably evaluated using the translational diffusion coefficients in the Stokes–Einstein equation, considering the high dilution (Accardo et al., 2013). The values are comparable to PC liposomes previously reported when the 100 nm filters are used for the extrusion process (Matsuzaki et al., 2000). Dimensions of all obtained liposomes are suitable for *in vivo* use. Results evaluation pushed to follow the *in vitro* experimentation for PC/Chol 80/20 formulation that has been selected for the good ratio in drug loading. Moreover, cholesterol ratio guarantees a right compromise able to assure at the same time indispensable rigidity and flexibility. Both properties are crucial parameters in the development of supramolecular aggregates acting as drug shuttle.

CDDP release and serum stability

Serum stability of CDDP-loaded liposomes, incubated with FBS/PBS (50/50) mixture, was assessed until 72 h.

Table 1. Drug loading content and structural parameters (hydrodynamic rays and PDI from dynamic light scattering measurements) for the different CDDP-loaded aggregates.

Samples	Drug loading content	Encapsulation efficiency (%)	R_H (nm) \pm S.D.	PDI
PC 100%	0.0058	0.68	59 \pm 40	0.287
PC/Chol 80/20	0.0076	0.71	55 \pm 30	0.217
PC/Chol 70/30	0.013	0.81	64 \pm 27	0.221

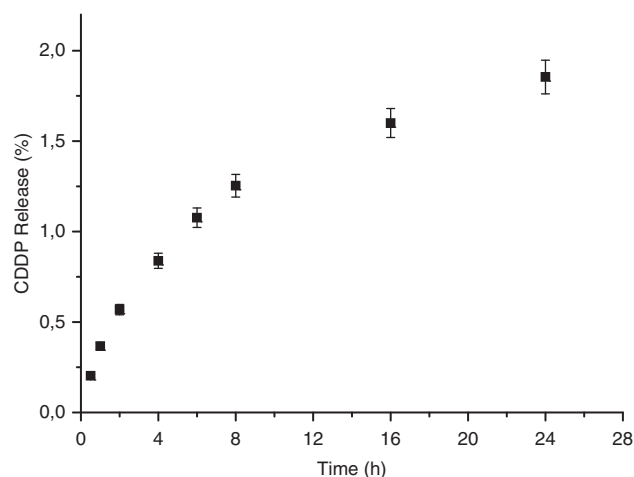


Figure 1. Release rate of CDDP from liposomes, performed in FBS/PBS (50/50) mixture. The amount of drug released (%) was estimated by ICP.

After incubation, size of the liposomal suspension was monitored by DLS measurements upon the time. The liposomal diameter did not show significant variations during the first 24 h of observation. After this period, a progressive increase in the size of the aggregates was observed, thus indicating an aggregation of the liposomes with the serum proteins (data not shown). Since the premature release of the CDDP from the carrier is an undesired event, due to the well-known severe toxic effects of free platinum drugs into the blood stream, the release kinetic of the CDDP from the liposomes in serum was evaluated. According to the serum stability results, CDDP release from the inner compartment of the liposomes was studied only until 24 h. A dialysis bag containing the liposomal suspension was dipped in a reactor containing 50% of fetal bovine serum in phosphate buffer, and placed at 37 °C under vigorous agitation for 24 h, and the amount of CDDP released was estimated by ICP-AES analysis. As shown in Figure 1, CDDP-loaded liposomes exhibit a low drug release rate: only 2% of the drug was slightly released in the first 10 h, suggesting that platinum containing liposomes present a very slow drug release.

Cellular studies

The growth inhibitory effect of both free and liposomal CDDP was evaluated in a panel of human tumor cell lines characterized by different CDDP sensitivity and containing examples of breast (MCF7, MDA-MB-231) and ovarian (SKOV-3, A2780) cancers. The panel contains ovarian cancer

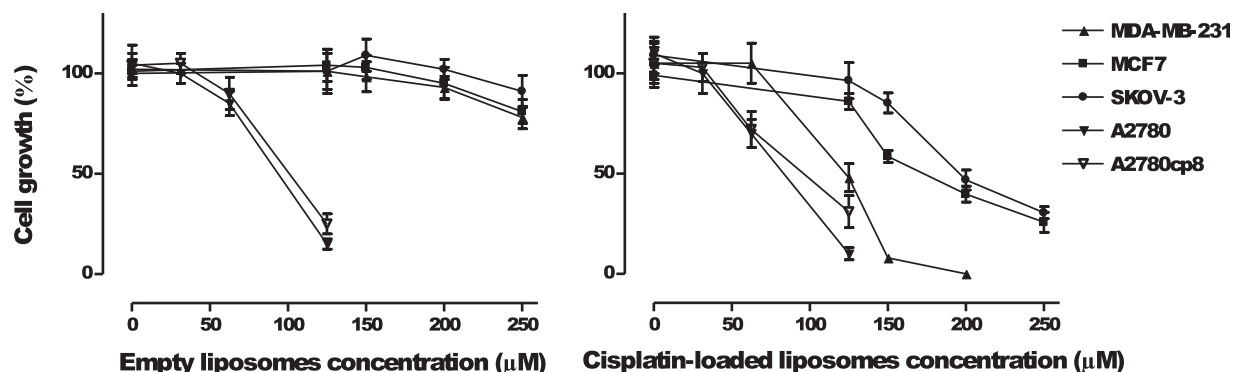


Figure 2. Cell growth data from SRB assays. Comparison of the potential inhibitory effect of the empty liposome (a) versus cisplatin-loaded in liposomes (b). Data are means \pm SD of three independent experiments performed in quadruplicate.

Table 2. Cell growth inhibitory activity of free CDDP, liposomal CDDP and empty liposome towards human tumor cells.^a

Cell lines	CDDP	Liposomal CDDP	Empty liposome
MCF7	4.5 \pm 0.7	2.6 \pm 0.6	>200
MDA-MB-231	8.8 \pm 1.2	1.56 \pm 0.25	>200
SKOV-3	6.0 \pm 1.1	3.36 \pm 0.87	>200
A2780	1.5 \pm 0.2	1.25 \pm 0.2	85.8
A2780cp8	7.4 \pm 2.1	1.4 \pm 0.3	94.9

^aIC₅₀ (mean value \pm SD calculated over at least three independent experiments) in μM (72 h treatment).

cells characterized by intrinsic (SKOV-3) or acquired (A2780cp8) resistance to CDDP. Results are presented in Figure 2 and Table 2.

After 72 h treatment, only a slight decrease of cell growth after incubating the cells with liposome concentrations $> 200 \mu\text{M}$ was evident for MDA, MCF7 and SKOV3 cell lines (Figure 2). In contrast, a similar inhibitory effect of empty liposome, was detected for both A2780 and A2780cp8 cell lines, the IC₅₀ being 85.8 and 94.9 μM , respectively.

A comparison of growth inhibitory activity of free CDDP, liposomal CDDP and empty liposomes is shown in Table 2. Across the cell lines, liposomal CDDP was more active than the free drug (mean IC₅₀ = 2.0 and 5.6, respectively), and the inhibitory potency was similar only for A2780 cells. Moreover, liposomal CDDP shows a narrower range of activity than CDDP, the most resistant/most sensitive cell line IC₅₀ ratios being 2.4 and 5.9, respectively. As expected, the A2780cp8 cell line was about 5-fold more resistant to CDDP compared to the A2780 cell line. In contrast, liposomal CDDP maintained its activity towards A2780cp8 cells, the resistance factor (RF, IC₅₀ A2780cp8/IC₅₀ A2780) being 1.1. However, a contribution of the liposome itself to the growth inhibition of the A2780/A2780cp8 pair cannot be excluded, as also suggested by the inhibitory effect data of the empty liposome as shown in Figure 2.

Liposomal CDDP was able to overcome CDDP resistance of ovarian A2780cp8 cells, which had been previously characterized for having a greater glutathione content (Behrens et al., 1987) and a reduced drug accumulation (Coluccia et al., 1999) with respect to the parental cell line. This property, along with the major activity of liposomal

CDDP towards tumor cells intrinsically resistant to CDDP (e.g. MCF7 and MDA-MB-231 cells) suggests a distinct liposome-mediated mechanism of action, perhaps not limited only to a drug accumulation improvement.

Conclusion

The current scenario about CDDP-loaded liposomes shows bright and dark features. Most of the classic preparations are based on saturated lipophilic tails inducing complications in platinum complex release and its consequential low cytotoxicity for tumor cells. Present study is a preliminary investigation of new CDDP-loaded liposomes based on Egg PC to address versus gynecological cancer cells. Structural data, loading studies and stability measurements allow to ensure stability of these systems. Liposomes fit within a critical size range (100–110 nm), the drug loading are comparable to SPI 077 formulations and the drug amount released within 10 h are suitable to their use *in vitro* and *in vivo*. The growth inhibitory effect of the 80/20 liposome formulation tested in a panel of cell lines of gynecological tumors shows promising results at least in the case of A2780cp8 ovarian cells.

Further studies will be carried out in order to introduce PEG chains in the hydrophilic shell of the liposome formulation, inducing blood circulation increase. Another improvement will be the functionalization with a bioactive peptide able to recognize receptors overexpressed in gynecological cancer cells.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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