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Research paper

Pharmacodynamics of cisplatin-loaded PLGA nanoparticles administered to tumor-bearing mice

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ABSTRACT

Biodegradable poly (lactic-co-glycolic) acid (PLGA) nanoparticles incorporating cisplatin have been developed to evaluate its *in vivo* efficacy in tumor-bearing mice.

In vitro study proved two mechanisms of action for cisplatin depending on the dose and the rate at which this dose is delivered. *In vivo* study, 5 mg/kg of cisplatin nanoparticles administered to mice, exhibited a tumor inhibition similar to free cisplatin, although the area under cisplatin concentration–time curve between 0 and 21 days (AUC_{0-21}) had lower value for the formulation than for drug solution ($P < 0.05$). This result was associated with a higher activation of apoptosis in tumor, mediated by caspase-3, after nanoparticles administration. Toxicity measured as the change in body weight, and blood urea nitrogen (BUN) plasma levels showed that cisplatin nanoparticles treatment did not induce significant changes in both parameters compared to control, while for free drug, a statistical ($P < 0.01$) increase was observed. In addition, a good correlation was found between time profiles of tumor volume and vascular endothelial growth factor (VEGF) plasma levels, suggesting that its expression could help to follow the efficacy of the treatment. Therefore, the PLGA nanoparticles seem to provide a promising carrier for cisplatin administration avoiding its side effects without a reduction of the efficacy, which was consistent with a higher activation of apoptosis than free drug.

1. Introduction

Cisplatin (CDDP) is widely used as a chemotherapeutic agent to treat a variety of solid tumors [1]. However, its therapeutic effect is dose limiting due to the presence of side effects such as gastrointestinal disturbance and specially nephrotoxicity [2]. In order to avoid the toxicity caused by its systemic administration, different types of controlled release formulation have been designed by several authors to enhance the drug levels in the tumor tissue [3–5]. Particularly, polymeric nano-sized carriers have shown a high tumor-targeting ability [5]. This preferential drug transport of these carriers is explained by the so-called "enhanced permeability and retention" effect, which is caused by the disorganized and increased vascularization of the tumor area [6,7]. Although, intratumoral administration is the best alternative to assure that the compound is effective, in most of the situations that is not possible. In this way, locoregional chemotherapy for adjuvant treatment of colorectal cancer seems to be a promising approach. In this malignant disease, patients have tumors that may spread with metastases in liver and peritoneal cavity [8]. In addition, the administration of the drug in the tumor area could help to increase its uptake due to the angiogenesis process. Vascular endothelial growth factor (VEGF) is a protein directly implicated in this process, especially in colorectal carcinoma. In fact, some authors suggest its expression as a useful marker to follow the tumor behaviour [9].

Controlled release formulations could be an emergent method to improve the delivery of the drug in the tumor target modifying the toxicities associated with a specific drug systemically administered [10]. However, the development of polymeric systems represents a serious problem in relation to the limitation for drug encapsulation. For cisplatin, this process shows several difficulties due to its physico-chemical characteristics, which make that to formulate systems in order to maintain adequate concentrations of this agent for 2 weeks or more, being very difficult [11]. In the last year, Moreno et al. [12] have reported a protocol to develop different PLGA systems for cisplatin using the emulsion solvent evaporation method. These systems were able to induce a different molecular mechanism in relation to the cell cycle, apoptotic factors and their cytotoxic profiles in comparison with free cisplatin in colon cancer cell line, suggesting that these formulations could play a significant role in the efficacy of cisplatin in this type of tumor. Thus, treatments with these formulations were able to induce cell

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arrest in the phase G2/M of the cell cycle followed by a gradual activation of caspase-3, the main apoptotic protein implicated in the cytotoxicity of cisplatin [13–15]. However, the cell arrest after high doses of free drug was in phase G1, which is associated, in part, with resistance phenomenon because some of these cells could be able to re-enter in a new cycle [15].

Therefore, in the present work, the objective was to explore the antitumor efficacy of PLGA nanoparticles incorporating cisplatin in tumor-bearing mice. That efficacy was related to the tumor size, the activation of apoptosis in tumor target and the presence of adverse effects. In addition, the correlation between the expression of VEGF protein and tumor size was also investigated.

2. Materials and methods

2.1. Materials

Poly(D,L-lactic-co-glycolic acid) (PLGA) polymer with a molecular weight (Mw) of 12,000 Da and a co-polymerization rate 50:50 (lactic/glycolic) (Resomer 502H) was purchased from Boehringer Ingelheim (Germany). Cisplatin (cis-diamminedichloride platinum (II)), polyvinyl alcohol (PVA 87–89% hydrolyzed, Mw 13,000–23,000) and Trizma hydrochloride were obtained from Sigma–Aldrich (Madrid, Spain).

2.2. CDDP nanoparticles preparation

PLGA nanoparticles (NP) loaded with cisplatin were prepared by a water–oil–water (w/o/w) emulsion solvent evaporation method [12]. A solution of cisplatin in Tris–HCl (1.67 mg/mL) was emulsified in 0.5 mL of chloroform containing 100 mg of PLGA, using a microtip probe sonicator (Microson XL 2000, Misonix Incorporated, USA) set at level 20 for 5 s. This primary (w/o) emulsion was added to 2 mL of PVA 9% containing 1 mg/mL of cisplatin and mixed by the Ultra-Turrax system (T 20 b, Ika Labortechnik, Germany) at 21,500 rpm for 20 s. The second w/o/w emulsion was transferred drop wise to 8 mL of PVA 9% saturated also with cisplatin (1 mg/mL) and agitated by magnetic stirrer for 3 h at room temperature (RT) until complete evaporation of the organic solvent. NP were collected by ultracentrifugation (40,000*g* for 10 min), washed with water, freeze-dried and stored at −20 °C until use.

To prevent the NP aggregation and retain their redispersibility after lyophilization, a suitable lyoprotectant (mannitol 6% [wt./vol.]) was added before lyophilization [16]. The developed process to formulate these nanoparticles was reproducible because in all preparations with 100 mg of polymer, the final amount of nanoparticles obtained was 72 ± 2.1 mg.

2.3. Characterization of nanoparticles

The size and zeta potential (ζ) of NP were determined by laser diffractometry using a Zetasizer Nano Series (Malvern Instruments, UK) after resuspension in 1 mL of PBS.

The morphology of NP was studied by scanning electron microscopy (SEM). Particles were attached to a double-sided tape and were spray coated with gold prior to inspection under electron microscope.

2.4. In vitro CDDP release profile

The loading of cisplatin in the NP was also quantified by high-performance liquid chromatography (HPLC) method. Weighed samples (5 mg) of NP were dissolved in 1 mL of NaOH (1 N) and kept at RT overnight in continuous agitation by a magnetic stirrer. Afterwards, the solutions were centrifuged at 18,000*g* for 10 min, and the supernatants were analyzed.

Samples of NP (5 mg) dissolved in 1 mL of PBS were placed in a microcentrifuge tube and maintained at 37 °C with constant stirring. At predetermined time intervals, between 0 and 35 days, an aliquot of the supernatant was collected and centrifuged (18,000*g*, 10 min) for analysis, and equal volume of fresh buffer was replaced. Samples were analyzed by HPLC following the method previously described by Moreno et al. [12]. Both studies were carried out by triplicate using three independent experiments.

2.5. In vitro cytotoxicity characterization

2.5.1. Antiproliferative effect

DHD/K12PROb adenocarcinoma colon cell line, obtained from BDIX rats, was grown as adherent monolayers in 25-cm^2 culture flasks at 37 °C in a 5% CO_2 humidified atmosphere and maintained in a mixture of Dulbecco's modified Eagle's and Ham's F-10 medium supplemented with 10% fetal bovine serum and 0.01% gentamicin.

Cells were seeded into 96-well microtiter plates at a density of 20×10^3 cells/well/200 μL and incubated in 5% CO_2 humidified atmosphere at 37 °C for 24 h. To evaluate the dependence of drug activity on concentration and exposure time, a design previously used in this cell line was followed [12]. The plates were treated with serial concentrations (2.5, 10, 18 or 50 μM) of free or encapsulated cisplatin (NP) and incubated for 3, 10, 24, 48, 72 and 144 h. After each treatment, the plates were washed twice with PBS, and the survival cells were quantified using the neutral red assay [17]. Optical density was read at 540 nm using a microtiter plate reader (Labsystems iEMS Reader MF). Wells with untreated cells were also included in each plate. Two linear standard curves, where the number of cells (from 5×10^3 to 100×10^3 and from 1×10^3 to 10×10^3 cells/well) were related to the absorbance measurements, were previously generated.

2.5.2. Cell cycle analysis

DHD/K12PROb cells (25×10^4 cells/well) were seeded into 6-well culture plates following the protocol described earlier. In each plate, wells with untreated cells (control) were also included. After each exposure time, the plates were washed with PBS, and the cells were collected in microtubes and washed by centrifugation (320*g*, 5 min). The pellets were incubated for 30 min at 37 °C, with 100 μL of Tween 20 (0.2% PBS) and 20 μL of ribonuclease type IIA (45 U/mL). Afterwards, the cells were stained with propidium iodide (25 μg/mL) for 10 min in darkness. The analysis of each sample by flow cytometry was performed with FACScan flow cytometer (Becton Dickinson) and CellQuest acquisition/analysis software.

2.5.3. Determination of VEGF and caspase-3 activity

Following the protocol described earlier, DHD/K12PROb cells (20×10^3 cells/well) were seeded into 96-well culture plates (MicroWell™ white-walled plates for luminescence). After each treatment, samples were split up in two parts: supernatants and pellets or cells.

VEGF levels were measured in the supernatant samples using the Quantikine Mouse VEGF Immunoassay Kit (R&D Systems, Inc., Minneapolis, USA) and following the manufacturer's protocol. Microtiter plate reader (Labsystems iEMS Reader MF) was used to measure the samples at 450–540 nm.

Caspase-3 activity was measured in the cell samples using the Caspase-Glo® 3/7 Luminometric Assay Kit (Promega Corporation, Madison, USA) [18]. Samples were washed twice with PBS and then mixed with the reagent provided by Promega, Caspase-Glo® 3/7 (100 μL per well) for 30 min. The RLU (relative light units) was quantified using a microplate Luminometer Orion II (Berthold sys-

tems, Germany). A standard curve with a commercial protein was built using the same kit assay. The final results were expressed as fg of caspase-3 activated per number of cells.

2.6. In vivo study

2.6.1. Animals and tumor implantation

Sixty-six female athymic nude mice weighing 20–25 g (aprox. 4 weeks old) were purchased from Harlam (Barcelona, Spain). Animals were housed in microisolator cages under positive-pressure ventilation and maintained in closed-shelf, laminar-flow racks to avoid contact with pathogens, odors or noises and kept under standard laboratory conditions. Sterilized food and water were available *ad libitum*.

To induce the tumor, 100 μL of PBS containing 2×10^6 DHD/K12PROb cells were subcutaneously injected into the right flank of the mice. Tumor growth and body weight were recorded daily. One week after cells injection and when the volume of the tumor was around 80 mm^3, animals were randomly divided into two main groups: Group I ($n = 24$) and Group II ($n = 36$), to characterize the efficacy and pharmacokinetics of cisplatin-NP.

The protocol of the study was approved by the Animal Experimentation Committee of the University of Navarra and is in accordance with the applicable European guidelines.

2.6.2. Antitumor efficacy of cisplatin-NP

Animals were randomly divided into four groups ($n = 6$/group) to receive the following treatments by intraperitoneal route: saline (Group 1 or control); 5 mg/kg of empty-NP (Group 2); 5 mg/kg of cisplatin in saline solution (Group 3) and 5 mg/kg of cisplatin-NP in saline solution (Group 4). All treatments were administered as a single dose once per week for two cycles.

The tumor volume (V) for each mouse was calculated using the formula: $V = AB^2/2$, where A and B correspond to the largest and the smallest diameter, respectively, measured with an electronic caliper. The terminal endpoint for animals was fixed to 1000 mm^3 as the maximum admitted volume, or when a decrease in a 20% of the body weight in relation to the weight just before starting the treatment, was achieved. Animals were sacrificed by cervical dislocation.

Blood samples (150 μL) from the retro-orbital plexus were collected before and at 6, 24, 48, 72 and 96 h after each treatment. Samples were centrifuged at 3000*g* for 10 min, and the plasma samples were stored at −20 °C until use.

Two different biomarkers were determined in each plasma sample: blood urea nitrogen (BUN) levels and vascular endothelial growth factor (VEGF) levels.

BUN levels were measured by the Quantichrom™ Urea Assay kit (BioAssay Systems, Hayward, CA, USA) following the manufacturer's protocol. A volume of 5 μL of plasma transferred in duplicate into a 96-well plate, was mixed with the working reagent (provided by the kit) and incubated for 30 min at RT. Afterwards, the absorbance in the plate was read at 540 nm using a microtiter plate reader (Labsystems iEMS Reader MF).

VEGF levels were quantified in 30 μL of each plasma sample using the Quantikine Mouse VEGF Immunoassay Kit (R&D Systems, Inc., Minneapolis, USA) following the manufacturer's protocol.

2.6.3. Pharmacokinetic study

Animals were randomly divided in two groups ($n = 18$/group) to receive 5 mg/kg of free cisplatin (Group 1) or 5 mg/kg of cisplatin-NP (Group 2) by intraperitoneal route once per week for two cycles. Blood samples (150 μL) were collected from the retro-orbital plexus at the following times: 2, 5, 15, 30, 60 min, 3, 6, 24, 48, 72, 96 and 168 h after drug administration, in heparinised capillary tubes, and immediately cooled on ice. In order to avoid suffering in the animal, each group was divided in six subgroups ($n = 3$) to obtain alternate samples. Plasma samples obtained by centrifugation at 3000*g* for 10 min at 4 °C, were stored at −20 °C until analysis. The tumor volumes and body weights were also recorded daily in these animals.

2.6.3.1. Tissue distribution. At the end of the experiments, all animals were sacrificed and spleen, lung, liver, kidneys and tumors were removed. Spleen, lungs, liver and kidneys were homogenized in acid-washed polyethylene tube using a solution of PBS (1 mL) with zirconia–silica beads (Bioespec Products, Bartlesville, OK). Each tube was shaken for 5 min at the maximum setting in the Mini-Beadbeater (Bioespec Products, Bartlesville, OK) followed by a centrifugation at 1100*g* for 10 min at 5 °C. The supernatant was incubated at 60 °C for 15 min and after that incubated at room temperature for 30 min; the sample was centrifuged at 1100*g* for 10 min to transfer the final supernatant to a polyethylene tube and kept at −20 °C until analysis.

In order to measure simultaneously in tumor, Pt levels and the activity of caspase-3, this tissue was incubated with 0.5 mL of trypsin for 90 min at 37 °C. Afterwards, this sample was mixed with 0.5 mL of fetal bovine serum (10%) and shook for 5 min at the maximum setting in the Mini-Beadbeater (Bioespec Products, Bartlesville, OK) followed by a centrifugation at 1100 g for 15 min at 5 °C. The supernatant was split up in two aliquots: one (50 μL) was used to quantify caspase-3 activated by the kit Caspase 3/7 Glo® (Promega, Spain), and the second one was incubated at 60 °C for 15 min and was used to quantify platinum analytically.

Cisplatin plasma and tissue concentrations were determined by graphite furnace atomic absorption spectrometry under the optimized operating conditions. The analytical system consisted of a Perkin Elmer Analyst 800 atomic absorption spectrometer (Norwalk, CT, USA) equipped with transverse-heated graphite atomiser, Zeeman background corrector and AS-800 autosampler. A platinum hollow cathode lamp (Perkin Elmer) operated at 30 mA was used to measure Pt at a wavelength of 265.9 nm with a slit width set at 0.7 nm. Time and temperature program for drying, charring and atomization in the graphite tube were assayed and optimized for a total volume of 50 μL [45 μL of sample mixed with 5 μL of Triton X-100 (0.5% v/v aqueous solution)].

The method was linear within the studied range of concentrations (20–750 ng/mL), and the accuracy of the assay was >98%. The limit of quantification was set at 42 ng/mL in undiluted plasma samples and at 54 ng/g in tissue samples.

2.7. Statistical analysis

All the results are expressed as the mean ± standard deviation (SD). Data were analyzed using the nonparametric Kruskal–Wallis test (for more than two groups) followed by the Mann–Whitney U-test (for two groups). Statistical significance was set at $P < 0.05$.

3. Results and discussion

3.1. Physico-chemical and morphological characterization

Table 1 shows that particle size formulated with the w/o/w method was approximately 450 nm. The PLGA polymer conferred a negative ζ-potential in the formulation, and no statistical differences were observed in the size and ζ-potential between loaded and non-loaded cisplatin formulations.

Fig. 1 represents the morphology of these particles obtained by SEM. The appearance of particles loaded and unloaded was indistinguishable because all of them were spherical with a similar diameter.

Table 1
Physico-chemical characteristics of cisplatin–PLGA-NP.

	Cisplatin-NP	Empty-NP
Size (μm)	0.45 ± 0.12	0.51 ± 0.09
ζ-Potential (mV)	−20.56 ± 0.65	−21.36 ± 1.08
Loading (μg drug/mg polymer)	6.74 ± 0.97	–
Drug concentration (μg drug/mL)	33.72 ± 4.86	–

Cisplatin-NP, cisplatin-loaded nanoparticles; empty-NP, unloaded nanoparticles.

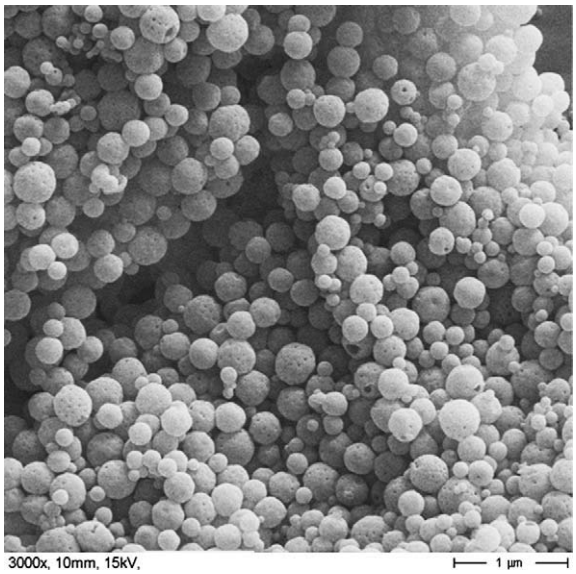

Fig. 1. Scanning electron micrographs of cisplatin–PLGA nanoparticles prepared with w/o/w method.

These results are in agreement with those previously reported by Moreno et al. [12]. These authors concluded that the particle size obtained with this method was dependent on the homogenization system used in the second emulsion. In this study, the Ultra-Turrax system led to obtain a particle size higher than the size obtained by them, when the microtip probe sonicator was used for the homogenization of two emulsions (180 nm ∅).

3.2. Encapsulation efficiency and in vitro cisplatin release

The encapsulation efficiency or loading, calculated as the amount of cisplatin recovered from each preparation in relation to the initial amount of cisplatin used for the fabrication process, was around 11% (w/w). This low encapsulation efficiency has been previously reported by other authors, who described the saturation of the second external phase with cisplatin, as a critical point during the formulation of different particles [19]. To obtain a loading efficiency around 10–12%, 1 mg/mL has been reported as the best saturation condition to prepare cisplatin particles with this method [12].

The values of drug loading were determined by using the direct method by NP degradation in NaOH and confirmed by the indirect method based on the *in vitro* release of cisplatin during 35 days.

The *in vitro* mean cumulative release profile of cisplatin from NP, represented in Fig. 2, involved an initial and rapid release phase followed by a second phase corresponding to a constant release during 3 weeks to finally reach a plateau. This profile was very similar to the profiles found by Avgoustakis et al. [19], Diez and Tros de Ilarduya, [20] and Duvvuri et al. [21] for different compounds encapsulated in PLGA particles, and was also similar to the profile assayed in cell culture medium (data not shown).

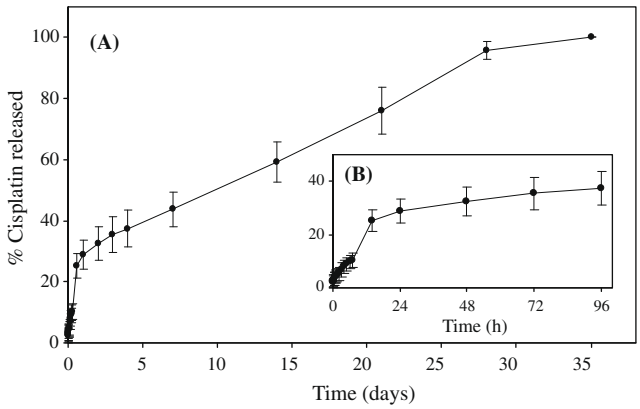

Fig. 2. (A) Cumulative "*in vitro*" release profile of cisplatin from NP in PBS at 37 °C. (B) The graph represents, in detail, the first times of this profile. Data represent the mean ± SD from three independent experiments ($n = 3$).

The percentage of drug released in the initial burst is dependent on the particle size when the compared particles, are formulated with the same polymer and for the same compound. Therefore, the value of 30% found in this work for the burst effect is according to those, 50% and 20%, previously reported for cisplatin encapsulated in PLGA (502H) particles with 0.18 and 9.2 μm in diameter, respectively [12]. In fact, this result for the burst effect, between 50% very rapid and 20% too slow, together with the size of the particle (nanoparticle) was the main characteristic to select this formulation for testing *in vivo*.

3.3. In vitro cytotoxicity characterization

3.3.1. Antiproliferative effect

The cytotoxic effect, characterized for different concentrations of free and encapsulated cisplatin after different time exposures, is represented in Fig. 3.

DHDK12-Prob cells treated with cisplatin free and encapsulated did not change the profiles of the cell growth curves during the first 10 h. However, after 24 h, the cytotoxicity was concentration dependent for both treatments. Additionally, this effect was for all concentrations statistically ($P < 0.01$) higher for free drug than for encapsulated drug. In fact, when these results were expressed as the IC_{50} [the concentration of cisplatin that is able to inhibit the 50% of the maximum antiproliferative effect] at 24 h, the values were very different: 18 ± 2.61 μM vs. 100 ± 4.15 μM for free and NP treatments, respectively. However, at 72 h, a significant reduction was found: 2.05 ± 0.67 μM vs. 8.12 ± 2.52 μM for free and NP, respectively. This result, previously reported [12], was explained by the presence of two effects on the polymer degradation, one cell-dependent and the other one time-dependent. In the case of cell-dependent effect, the presence of PVA in the formulation plays a significant role providing a cell adhesion in the polymeric structure and then inducing a rapid matrix degradation [22]. The time dependence is described as an intrinsic effect of cisplatin.

Control (untreated) group and the group treated with empty-NP (cells treated with equal amount of polymer that it was used for 50 μM) displayed the same behaviour, since no statistical differences were observed among them. This result was expected because PLGA is a biodegradable polymer, approved by FDA for human applications.

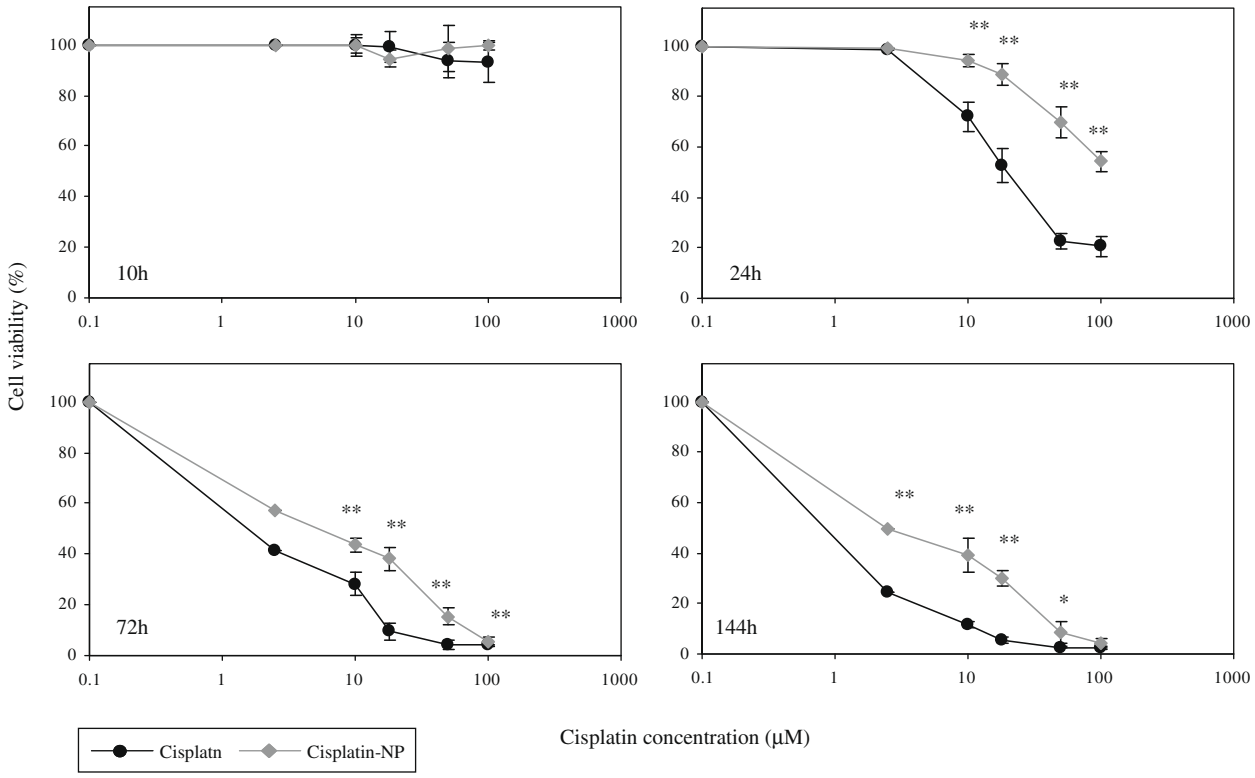

Fig. 3. Antiproliferative effect of cisplatin free and encapsulated in NP after different concentrations and exposure times. The effect is expressed as the percentage of survival cells in relation to the corresponding control. Data represent the mean ± SD from three independent experiments ($n = 3$). Free cisplatin treatment (●) and NP (♦). $^{*}P < 0.05$, $^{**}P < 0.001$.

3.3.2. Cell cycle analysis

The distribution of the cell cycle shows significant modifications in relation to the treatments with free and encapsulated cisplatin, as is shown in Fig. 4. For cisplatin, two different behaviours were observed depending on the type of administration, in solution or encapsulated. For NP, a progressive increase in cells arrest in G2/M was induced by all concentrations, reaching a peak at 48 h of drug exposure. Similar arrest was observed for low concentrations of free drug, although the maximum was at 144 h. However, the higher concentrations (18 and 50 μM) of free cisplatin induced cells accumulations in G0/G1, decreasing very quickly the G2/M phase, especially at 50 μM. This result was consistent with a dual mechanism of cell death, which was very rapid for higher doses of free cisplatin associated with mechanisms of resistance and significant side effects, while for lower doses, cell death was at later times and associated with an effective apoptosis [12,23]. In fact, a single high dose is the most common regimen of administration in patients treated with this compound and is related to the presence of irreversible adverse effects, such as nephrotoxicity and ototoxicity. For this reason, the mechanism induced by the NP could be a good strategy to test its *in vivo* efficacy. In addition, although this study was carried out in unsynchronized cells, all treatments with NP led to reach a maximum of cell accumulation in G2/M at 48 h, suggesting an effective cell arrest due to this continuous delivery.

3.3.3. Determination of VEGF and caspase-3 activity

The cell arrest on the cell cycle is, in general, followed by cell death. Mueller and co-workers [24] found that cell death after cisplatin treatment did not occur without prior G2/M arrest in the cell cycle. In this phase, where the damage of DNA is usually lethal, the apoptotic process is initiated and executed. Taking into account that caspase-3 is reported as the main apoptotic factor activated by cisplatin [13,14,25], this protein was quantified after different treatments. Fig. 5 shows that caspase-3 activity increased in a gradual manner with NP, reaching a peak at 72 h. For free cisplatin, the activation of this protein was different depending on the concentration, because for low concentrations was gradual over time, whereas for higher (18 and 50 mM) was rapid and intensive. Since the cisplatin concentration released from NP is very low (30% at 24 h), a low activation of caspase-3 is expected although the arrest in G2/M confirms the induction of apoptosis. In fact, Mueller et al. [24] showed that cells were more sensitive to cisplatin treatment in this phase of the cycle than in G1.

On the other hand, VEGF is a potent and specific mitogen for endothelial cells that activates the angiogenic switch *in vivo* and enhances vascular permeability in tumor areas [26,27]. Table 2 represents the correlation between cell viability and VEGF levels expressed in the medium. The changes in each parameter were calculated in relation to the corresponding control at each time and then expressed in percentage. These percentages were very similar assuming that VEGF might be a suitable marker for testing the drug effect, even when this factor is very sensitive to the environmental conditions. Therefore, this result represents a new strategy to study the time evolution of the antiproliferative effect of a treatment, avoiding the lysis of the cells.

3.4. In vivo study

3.4.1. Antitumor efficacy of cisplatin-NP

In a pilot study, a range of doses between 2.5 and 20 mg/kg was tested in mice to evaluate the number of death over time. The selection of the schedule was based on the minimum dose required to reduce the tumor volume, consisting on 5 mg/kg for cisplatin. This dose was adjusted to 5 mg/kg of cisplatin basis for NP. The treatment was administered by intraperitoneal route once a week

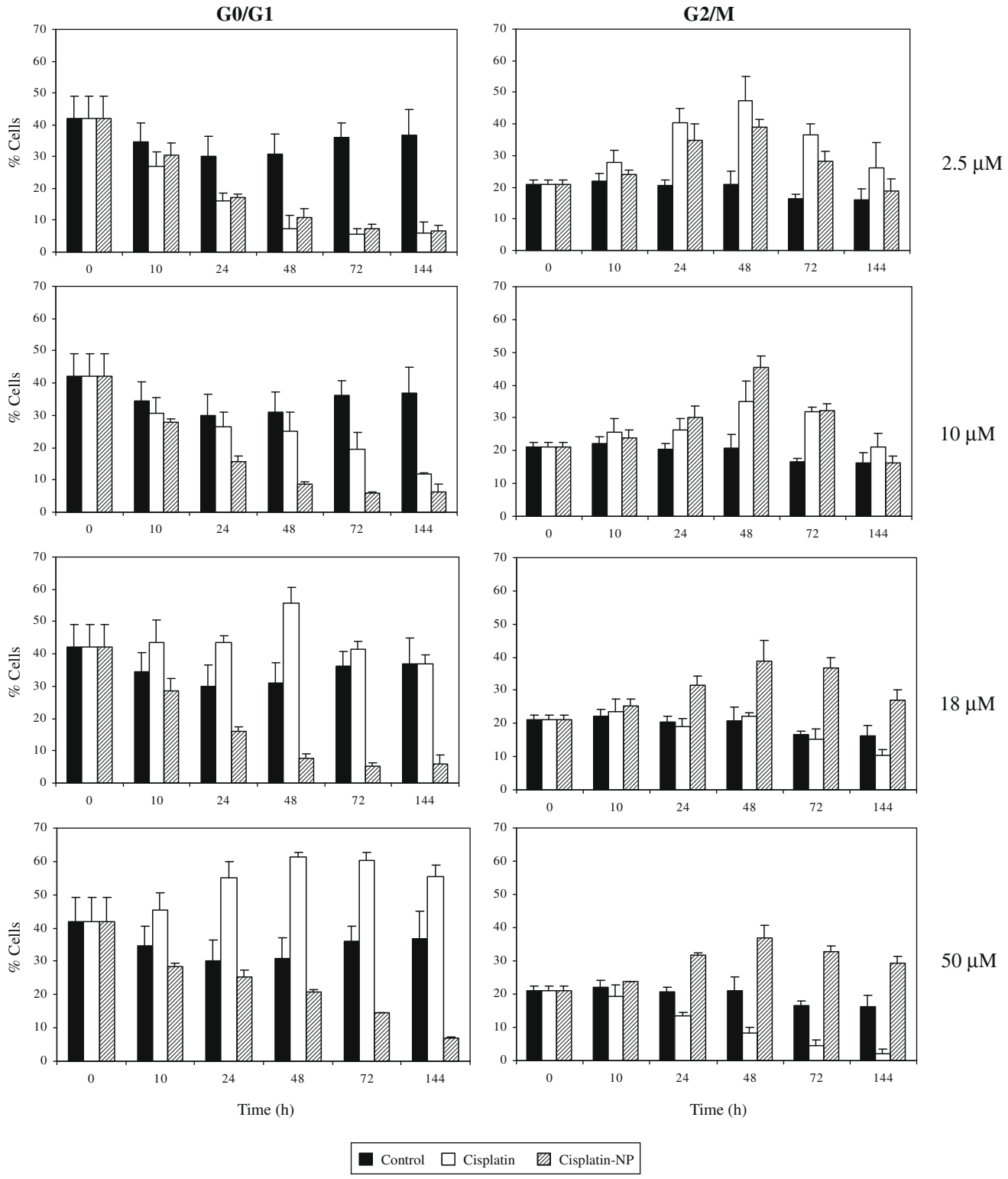

Fig. 4. Cell cycle distribution after different treatments with free and encapsulated cisplatin. Data represent the mean ± SD from three independent experiments ($n = 3$).

for 2 weeks. All treatments effectively inhibited tumor growth and displayed similar response because of no statistical differences were found among them (Fig. 6A). However, the statistical difference ($P < 0.001$) was found between the treated groups and the control group. The tumor volume for treatments had a relatively slow increase attaining only 200 mm^3, whereas in control group, tumor cells grew rapidly reaching 900 mm^3. Neither saline nor empty-NP treatments had any measurable effect on tumor growth showing similar time profiles.

On the other hand, 5 mg/kg is a dose within the range (2.5–20 mg/kg) used by other authors to study tolerance and antitumor activity of different cisplatin formulations. For example, Kim et al. [5] have studied the efficacy of 10 mg/kg cisplatin-NP administered intravenously once every 3 days for 15 days. The authors found

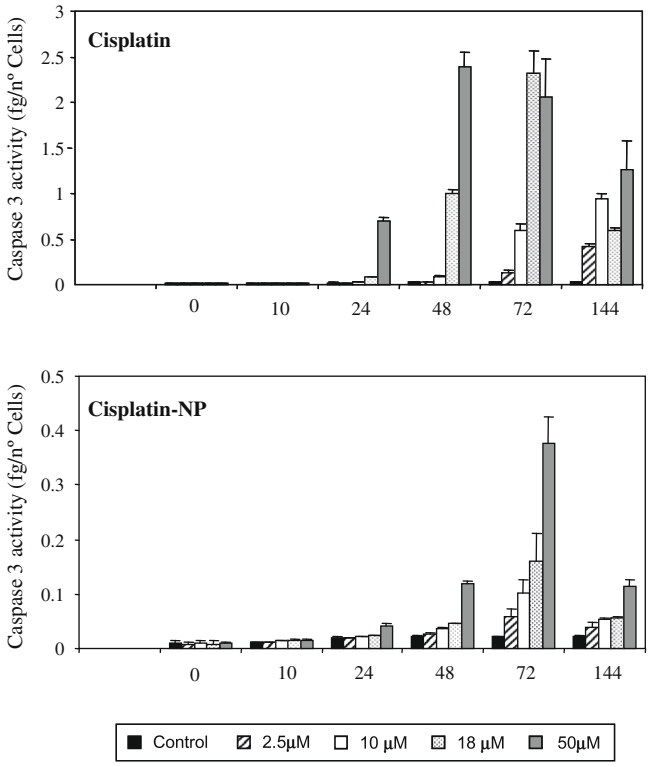

Fig. 5. Levels of caspase-3 activated vs. time in each group of treatment. Data represent the mean ± SD from three independent experiments ($n = 3$).

that the formulation was able to inhibit the tumor growth, accumulating Pt in that tissue. However, certain degree of toxicity was present because some animals died during the study. The dose schedule, 5 mg/kg once per week, used in the present study, was able to control the growth of the tumor keeping their volume between 100 and 200 mm^3 and to allow also that all animals completed the study. This result is according to those previously reported by Mattheolabakis et al. [28], where 5 mg/kg of cisplatin loaded in PLGA-mPEG nanoparticles administered once a week was a dose well tolerated in relation to animal survival and body weight changes, although the efficacy was reduced in comparison with free cisplatin. In fact, sometimes with this technology, the efficacy is not improved, but the toxicity is reduced leading to a more effective drug combinations.

On the other hand, VEGF plasma levels measured in all animals showed a similar time profiles than those obtained for tumor growth (Fig. 6B). The displayed similarity between VEGF and tumor growth curves suggests that VEGF could be used as an adequate biomarker to follow the antitumor efficacy of the treatments, as was observed in the previous results from the *in vitro* study. This protein, associated with the neovascularization of the tumors, represents a new target in the cancer therapy, combining antitumor drugs with anti-VEGF to inhibit the vascularization which can enhance the permeability in this tissue [7]. Although the prognostic significance of VEGF expression in colon cancer is still unclear, it seems that different levels of these factors have been associated with different stages of the tumor, following the Dukes's classification [9]. The authors found a correlation between the intensity of the VEGF expression and the clinical status in patients with this type of cancer. Therefore, the findings about the VEGF plasma levels observed in this work support the idea that could be a very useful tool to study the efficacy of different therapeutic strategies.

The changes in body weight of mice after cisplatin treatments are depicted in Fig. 7. The analysis of these variations is defined as an adverse effect of a therapeutic regimen. Cisplatin-NP demonstrated favourable results without any loss in the body weight, whereas free drug induced a nearly 10% of the decrease ($P < 0.01$ vs. control). No significant differences were observed between mice treated with saline or empty-NP.

The BUN plasma levels reported as a marker related to platinum toxicity were measured during the first cycle of treatment. These values showed a statistical ($P < 0.01$) increase in animals treated with free drug compared to control animals or animals treated with cisplatin-NP, as is represented in Table 3. The toxicity caused by free drug, previously reported by other authors [29], was observed for, at least, 72 h after administration. Based on these results, it is possible to conclude that cisplatin–PLGA-NP showed a significant antitumor efficacy, as well as a reduction of the side effects in relation to free cisplatin.

3.4.2. Pharmacokinetic study

To evaluate the cisplatin distribution, a dose of 5 mg/kg i.p. of free and encapsulated form was administered to tumor-bearing mice. Cisplatin plasma levels were quantified during two cycles of treatment as is represented in Fig. 8. The descriptive pharmacokinetic parameters such as C_{max} (maximum value of plasma concentration), T_{max} (time at the C_{max}) or AUC (area under plasma concentrations curve) calculated by the linear trapezoidal method using the mean of the concentrations for each of the cycle are shown in Table 4. A statistical difference was found in the AUC_{0-21} for cisplatin after NP administration in comparison with free drug ($P < 0.05$). In fact, the value of C_{max} for NP was the only 3% of the C_{max} for free drug. This result was expected because the

Table 2
VEGF levels measured in the cell culture medium at 24 and 72 h after different treatments of free cisplatin and cisplatin-NP. VEGF (%) represents the change expressed in percentage, between each treatment and the control, and viability (%) represents the results from the antiproliferative experiment. Data represent the mean ± SD ($n = 3$ independent experiments).

	24 h			72 h		
	VEGF (pg/mL)	VEGF (%)	Viability (%)	VEGF (pg/mL)	VEGF (%)	Viability (%)
Cisplatin						
Control	414. 3 ± 12.7	100	100	911.0 ± 7.4	100	100
2.5 μM	375.0 ± 37.0	90.5 ± 9.0	98.5 ± 6.1	445.2 ± 26.8	48.9 ± 3.0	50.8 ± 6.4
10 μM	292.7 ± 19.8	70.7 ± 4.9	71.9 ± 6.7	268.9 ± 33.6	29.5 ± 3.7	28.1 ± 3.4
18 μM	224.1 ± 21.8	54.1 ± 5.4	52.6 ± 2.9	116.4 ± 17.5	12.8 ± 2.0	9.5 ± 1.7
50 μM	109.3 ± 14.3	24.5 ± 3.6	22.8 ± 3.7	38.2 ± 13.2	5.2 ± 1.5	4.1 ± 0.5
Cisplatin-NP						
Control	404.1 ± 16.1	100	100	918.1 ± 10.7	100	100
2.5 μM	415.7 ± 21.5	102.9 ± 6.4	99.1 ± 2.2	515.0 ± 27.5	56.1 ± 3.0	57.3 ± 2.8
10 μM	405.7 ± 22.7	100.4 ± 5.7	94.2 ± 4.2	384.5 ± 19.8	41.9 ± 2.2	43.6 ± 4.7
18 μM	369.3 ± 19.3	91.4 ± 4.9	88.8 ± 6.2	331.6 ± 17.3	36.1 ± 1.9	38.0 ± 3.3
50 μM	301.8 ± 21.8	74.7 ± 5.5	69.9 ± 4.0	153.4 ± 16.9	16.7 ± 1.9	15.3 ± 1.8

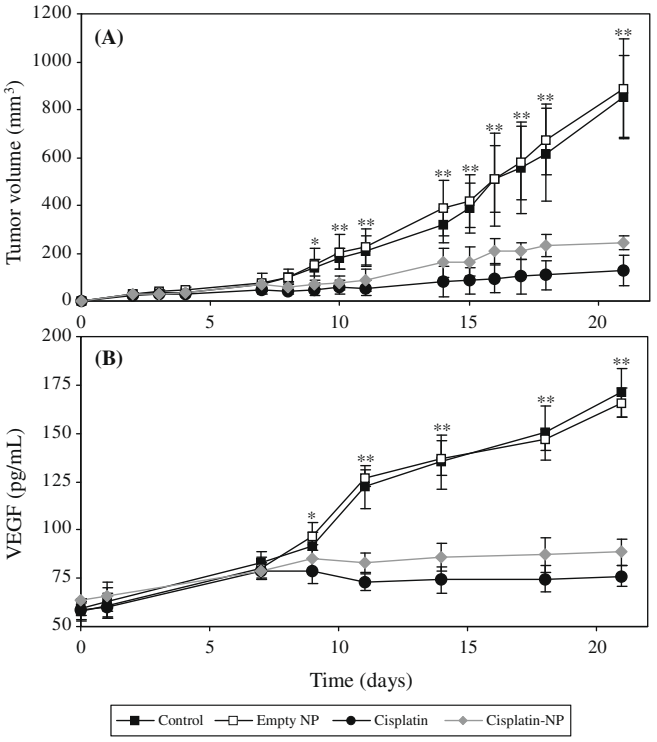

Fig. 6. (A) Time profile of the tumor volume and (B) plasma VEGF levels after saline (control), empty-NP, free cisplatin and cisplatin-NP. Each point represents the mean ± SD of six animals. $^{*}P < 0.05$, $^{**}P < 0.001$, each treatment vs. its corresponding control.

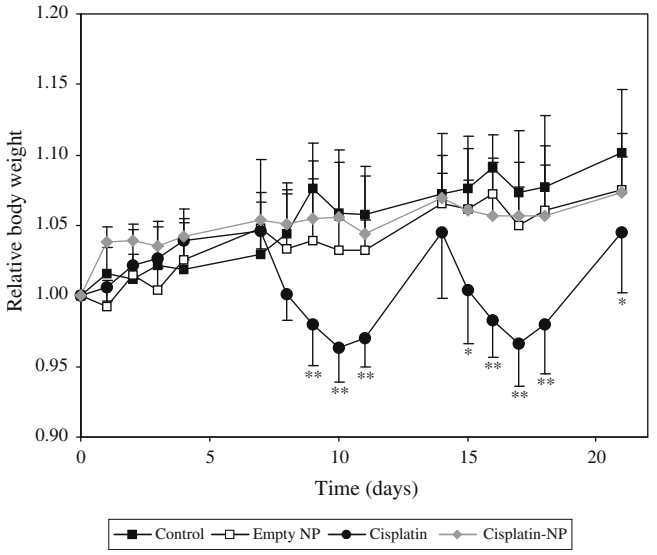

Fig. 7. Body weight changes in mice treated with saline (control), empty-NP, free cisplatin and cisplatin-NP. Each point represents the mean ± SD of six animals. $^{*}P < 0.05$, $^{**}P < 0.001$, free cisplatin vs. control.

administration of this type of PLGA-NP is associated with lower C_{max} values, longer T_{max} and higher MRT (mean residence time) and volume of distribution [30].

Antitumor effect is described to be proportional to AUC [31]; however, in this case, no statistical difference was observed between both treatments (NP vs. free drug) in the area under effect curve ($AUEC_{0-21}$), calculated by the linear trapezoidal method using the mean value of the effect for each of the cycles. The high

Table 3
BUN plasma levels (mg/dL) obtained after saline (control), empty-NP and 5 mg/kg of free cisplatin and cisplatin-NP, respectively. Data represent the mean ± SD of six animals.

Time (days)	Groups			
	Control	Empty-NP	Cisplatin	Cisplatin-NP
Basal	20.1 ± 1.6	19.8 ± 3.3	21.1 ± 1.2	20.0 ± 0.6
7	20.3 ± 1.5	21.7 ± 1.9	18.5 ± 2.6	19.6 ± 1.7
7.25	21.3 ± 2.9	22.2 ± 0.7	27.5 ± 1.2*	20.0 ± 0.6
8	20.1 ± 2.5	21.8 ± 2.1	42.1 ± 3.2**	19.9 ± 1.9
9	21.5 ± 2.4	21.4 ± 2.3	35.7 ± 3.9**	20.3 ± 2.1
11	21.3 ± 1.3	21.5 ± 0.8	20.9 ± 1.5	19.6 ± 0.4

* $P < 0.05$.
** $P < 0.001$, in relation to control group.

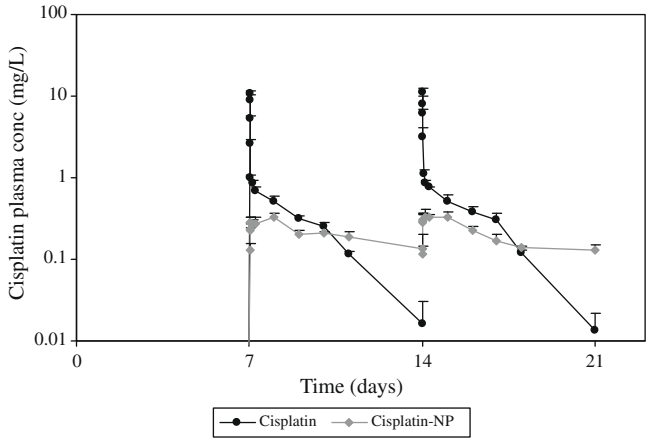

Fig. 8. Time profiles of total plasma concentrations of cisplatin after the intraperitoneal administration of 5 mg/kg of cisplatin free and encapsulated. Each point represents the mean ± SD of three animals.

Table 4
Descriptive pharmacokinetic parameters and the area under antitumor effect curve.

	Cisplatin	Cisplatin-NP
AUC_{0-21} (mg day/L)	3.77 ± 0.33	2.80 ± 0.02*
$AUCE_{0-21}$ (mm^3/day)	1327.95 ± 674.74	2200.17 ± 215.72
T_{max} (min)	5	60
C_{max} (mg/L)	10.83 ± 0.95	0.33 ± 0.05

AUC_{0-21}, area under curve of cisplatin plasma concentrations between 0 and 21 days; $AUEC_{0-21}$, area under curve of the antitumor effect induced by cisplatin between 0 and 21 days; T_{max}, time for the C_{max}; C_{max}, maximal plasma concentration of cisplatin.
* $P < 0.05$, free cisplatin vs. cisplatin-NP.

interindividual variability found after free drug administration could be one of the reason.

In this study, the i.p. administration of free drug was followed by a rapid absorption into systemic circulation, as is represented in Fig. 8. This result is according to the fact that high concentrations in the peritoneum reach (within 5 min) high plasma concentrations very quickly leading to important systemic side effects [32,33].

In the case of NP, cisplatin plasma concentrations increased during the first hour after administration, reaching a plateau for 24 h and declining very slow over a week. The difference in cisplatin plasma levels between free and encapsulated administration was reflected also in tumor, as is represented in Table 5. Nevertheless, the formulation was able to induce an apoptotic effect, measured by the activation of caspase-3, higher than free drug. The increase in this activation, calculated in relation to the control

Table 5
Total cisplatin concentrations in different organs, lung, liver, kidneys, Spleen and tumor after i.p. administration of 5 mg/kg/two cycles of free and encapsulated. Activity of caspase-3 induced in tumor.

	Drug levels (μg cisplatin/g tissue)					Caspase-3 (pg/g tissue)
	Lung	Liver	Kidneys	Spleen	Tumor	Tumor
Control	n.a.	n.a.	n.a.	n.a.	n.a.	21.2 ± 12.3[a]
Cisplatin	0.4 ± 0.2	3.6 ± 1.3	5.1 ± 1.4	2.3 ± 1.4	1.1 ± 0.6	46.1 ± 26.0[b]
Cisplatin-NP	0.4 ± 0.1	2.1 ± 0.9	5.9 ± 1.9	6.5 ± 1.8*	0.12 ± 0.06*	98.8 ± 23.8*,a,b

n.a.: no applicable.
Data represent the mean ± SD (n = 18).
* $P < 0.05$.
[a] Statistical differences were calculated for drug concentration between free and encapsulated (NP) treatments; and for apoptotic activity, between NP and saline.
[b] Statistical differences were calculated for drug concentration between free and encapsulated (NP) treatments; and for apoptotic activity between NP and free drug.

group, was 4.7-fold for NP, while for free drug, it was only 2.9-fold. The control group has to be taken into account because in this group, some physiopathological conditions are able to induce apoptosis. The discrepancy between cisplatin tumor levels and apoptotic activity could be explained by the sustained release of NP, which displayed a mechanism of action to induce apoptosis different than free drug, as was observed in *in vitro* results. Evidence about this type of behaviour has been reported by Kishimoto et al. [34]. They found that after intermittent administration of very low doses of cisplatin, the activity of caspase-3 was similar or even higher than after a single high dose.

In addition, the diffusion of cisplatin into the core of the tumor has serious difficulties needing a high concentration or a long period of time to induce cell death in the center of the larger tumors [32].

On the other hand, the intraperitoneal route simulates a locoregional administration easier to use than intratumoral way and allows also a decrease in the systemic toxicity [8]. In this case, the main organ implicated in NP uptake was the spleen due to the action of the peritoneal nodules. No accumulation was observed in lungs or liver. In the case of the kidneys, where tubular necrosis may occur [35], the cisplatin levels observed for both treatments were similar; however, the nephrotoxic effect for NP was minimum (Table 3). This observation could be explained by the continuous release of drug from the NP allocated in the spleen [31].

4. Conclusion

In the present study, we describe the development of a PLGA nanoparticle for cisplatin using the method w/o/w. This formulation was able to combine a significant efficacy, measured as a reduction of tumor volume and an activation of apoptosis, with a reduced toxic effect, represented by the change in body weight of mice and BUN plasma levels. Finally, the expression of VEGF showed a good correlation with the efficacy of the treatment in both situations, *in vitro* and *in vivo*, opening the controversy about its study as a useful biomarker, in this type of tumor.

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