Available online at www.sciencedirect.com
ScienceDirect
European Journal of Pharmaceutics and Biopharmaceutics 68 (2008) 503–512
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Characterization of cisplatin cytotoxicity delivered from PLGA-systems

Daniel Moreno [a], Conchita Tros de Ilarduya [a], Eva Bandrés [b], María Buñuales [a], María Azcona [a], Jesús García-Foncillas [b], María J. Garrido [a,*]

[a] Department of Pharmacy and Pharmaceutical Technology, University of Navarra, Pamplona, Spain
[b] Center for Applied Medical Research (CIMA), University of Navarra, Pamplona, Spain

Received 9 February 2007; accepted in revised form 1 August 2007
Available online 8 August 2007

Abstract

Biodegradable lactic acid-glycolic acid copolymer (PLGA) formulations incorporating cisplatin have been developed to evaluate the cytotoxicity of this agent in cultured cells. Two different W/O/W protocols were used to formulate micro- (MP) and nanoparticles (NP) under the solvent evaporation method. Although the amount of cisplatin encapsulated was higher in the MP, the efficiency of encapsulation was similar: 10.33% vs. 11.23%, for both MP and NP, respectively. The "in-vitro" release profiles displayed a significant difference in the initial burst effect, which had a significant impact in the antiproliferative effect of cisplatin. In addition, a duality in the cell cycle distribution was found for both formulations and low doses of free cisplatin (2.5, 10 μM) in comparison with the high doses of free cisplatin. The 50 μM caused a rapid inhibition of cells growth followed by a significant loss of cells in phases G0/G1 and G2/M which correlated with an increase in the number of cells in sub-G1. However, cisplatin released from controlled formulations induced an accumulation of cells in the phase G2/M. These results led to enhance the caspase-3 activity for MP and NP. These findings indicate that controlled release formulations of cisplatin are able to induce a more effective apoptosis than free cisplatin.

Keywords: Poly(D,L-lactic-co-glycolic acid) (PLGA); Microparticles; Nanoparticles; Cisplatin; Drug release; Cytotoxicity; Apoptosis

1. Introduction

The new drug delivery systems, such as polymeric particles, have been considered promising carriers for anticancer drugs [1–3]. However, the encapsulation in these systems highly depends on several factors such as the polymer physicochemical characteristics and the method of preparation. Cisplatin, classified as a potent anticancer agent in many solid tumors, has a poor activity in colorectal cancer. In general, its limited efficacy due to the development of resistance. The resistance mechanism is not fully understood, although the reason is probably multifactorial [4]. Therefore, to counteract resistance, clinical dosing will generally be elevated to higher levels, however this type of administration is associated with serious side effects, including renal and auditory disturbances, nausea and vomiting [5,6]. To localize the delivery of cisplatin to the tumor, some specific approaches have been evolved, particularly the microspheres and liposomes [7–9]. Nevertheless, polymeric delivery systems are commonly used for entrapping different bioactive substances such as vaccines, proteins, DNA and RNA because in general, these systems can reduce the toxicity and increase the activity of these substances [10,11]. Although there are several types of polymers, the poly(lactic-co-glycolic) acid (PLGA) is the most widely used due to its biocompatible and biodegradable properties [3]. In this way, the use of these systems in general, and specifically in the case of cisplatin, allow a sustained drug release increasing drug effects and reducing side effects [2,12,13]. Some studies on the preparation of cisplatin-loaded microparticles have identified the presence of various difficulties caused by the physico-chemical characteristics of this compound. For example, Fujiyama et al.

* Corresponding author. Department of Pharmacy and Pharmaceutical Technology, School of Pharmacy, University of Navarra, 31080 Pamplona, Spain. Tel.: +34 948 425600 6529; fax: +34 948 425649.
E-mail address: mgarrido@unav.es (M.J. Garrido).

0939-6411/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2007.08.006

[2] concluded that the preparation of sustained-release microparticles that maintain adequate concentrations of this agent for 2 weeks or more, is very difficult. However, even in that situation, these strategies might improve the therapeutic index of cisplatin, increasing the drug delivery selectively to tumor tissue, brain, gastric area, ovarian and so on.

On the other hand, data from the literature reported that continuous infusion or multiple administrations of low-doses was an excellent regimen for inducing apoptosis more effectively than a single high-dose exposure of cisplatin [6]. This evidence supports the idea that the sustained release formulations could be a good system for increasing therapeutic efficacy or possibly overcoming the resistance phenomenon, as Koziara and co-workers [14] demonstrated in the case of paclitaxel used to treat colorectal tumors.

The objective of the present work is therefore to develop different protocols to formulate nano- and microparticles entrapping cisplatin and to evaluate the influence of these systems on the cytotoxicity of this anti-tumor agent in cultured cells.

2. Material and methods

2.1. Materials

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

2.2. Microparticles preparation

PLGA microparticles (MP) loaded with cisplatin were prepared by a water- oil- water (w/o/w) emulsion solvent evaporation method following the specifications employed by Diez and Tros de Ilarduya [11], with some modifications. Briefly, 1.67 mg/mL of cisplatin dissolved in 30 mM Tris–HCl (pH 7.5) was emulsified in 1.5 mL of chloroform containing 100 mg of PLGA using an Ultra-Turrax (T 20 b, Ika Labortechnik, Germany) at 13,500 rpm for 10 s. This w/o emulsion was added to 3 mL of PVA 9% (w/v) containing 1 mg/mL of cisplatin and emulsified using again an Ultra-Turrax (13,500 rpm/10 s). The resultant w/o/w emulsion was transferred dropwise to 7 mL of PVA 9% saturated also with cisplatin, and agitated by a magnetic stirrer for 3 h at room temperature (RT) until complete evaporation of chloroform. The microparticles were collected and purified by applying three cycles of centrifugation (40,000*g*, 10 min) and washing with distilled water. Finally, the microparticles solution was lyophilizated and stored at −20 °C until use.

2.3. Nanoparticles preparation

The nanoparticles (NP) loaded with cisplatin were prepared using also the double emulsion method (w/o/w) developed by Avgustakis et al. [1] incorporating significant modifications from Díez and Tros de Ilarduya [11]. An aqueous solution of cisplatin (2.5 mg/mL in a volume of 0.4 mL) was emulsified in 2 mL of dichloromethane 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 transferred to 6 mL of PVA 9% solution containing 1 mg/mL of cisplatin and the mixture was probe sonicated at level 20 for 10 s. The w/o/w emulsion was agitated by a magnetic stirrer for 3 h at RT until complete evaporation of the organic phase. The nanoparticles were collected by ultracentrifugation (40,000*g* for 1 h), washed three times with distilled water, freeze-dried and stored at −20 °C until use.

2.4. Characterization of micro- and nanoparticles

The size and zeta potential (ζ) of micro- and nanoparticles were determined by laser diffractometry using a Zetasizer Nano Series (Malvern Instruments, UK) after resuspension in phosphate buffered saline (PBS), pH 7.4 (1 mL). All measurements were performed in triplicate.

2.5. Cisplatin loading and "in-vitro" release study

The loading of MP and NP with cisplatin was determined using both a direct and an indirect procedure. In the direct method, 5 mg of lyophilized particles were dissolved in 1 mL of NaOH (0.1 N) and kept at RT overnight in agitation using a magnetic stirrer. Afterwards, the solution was centrifuged at 18,000*g* for 10 min and 90 μl of the supernatant was used to quantify cisplatin by high-performance liquid chromatographic technique (HPLC). The "in-vitro" release study was used for the indirect method. Cisplatin-loaded PLGA-micro- and nanoparticles (5 mg) were suspended in 1 mL of phosphate buffered saline (PBS). The suspension was placed into a microcentrifuge tubes and maintained at constant stirring in a water bath at 37 °C. At predetermined time intervals, the tubes were centrifuged (18,000*g*, 10 min) and the supernatants collected for HPLC analysis. After sampling, the incubation medium was replaced by fresh PBS and the tubes were placed back in the incubator.

To quantify the release kinetics of cisplatin from the two formulations, a HPLC method was developed. This method is based on other previously reported by Augey et al. [15] and López-Flores et al. [16] with some modifications. In brief, an aliquot of 90 μl of the supernatant collected from "in-vitro" release studies, was placed in a 1.5-mL eppendorf tube and mixed with 10 μl of a solution of sodium diethyldithiocarbamate (DDTC) (10% [wt/vol] prepared with 0.1 N NaOH). Samples were incubated at

37 °C in a water bath for 1 h and cooled on ice for 10 min to stop the reaction (formation of chelates cisplatin-DDTC). These chelates were extracted with 100 μl of chloroform by vortexing at maximal speed for 1 min and centrifuged at 1000*g* for 5 min at 5 °C. Then, 10 μl of the chloroform layer was injected into the chromatographic system which consisted of a Hewlett Packard HPLC HP 1100 equipped with a quaternary pump, autosampler and a UV detector.

The analytical separation was performed at 30 °C by a Kromasil C-18 (25 × 0.46 cm i.d. of 5 μm particle size). The mobile phase fixed at a flow rate of 1 ml/min was a mixture of methanol/water (25:75). Detection was performed at 254 nm.

Retention times of 8 and 9 min were found for DDTC and DDTC-Pt complex, respectively. The assay was calibrated by standards prepared following the procedure described above. The accuracy of the assay was >90%. The method was linear within the concentration range studied (100–0.2 μg/mL) and the limit of quantification was considered 0.2 μg/mL.

2.6. "In-vitro" culture cells study

DHD/K12PROb cell line obtained from a colon adenocarcinoma, induced in syngenic BD-IX rats, was used in this study. The cells were 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 separated into a single-cell suspension in culture medium by trypsinization and seeded at a density of 20×10^3 cells/well/180 μl into 96-well culture plates. These plates were incubated in 5% CO_2 humidified atmosphere at 37 °C for 24 h. After this period of time, the plates were treated with serial concentrations of microparticles, nanoparticles or free cisplatin (2.5, 10, 18, 50 and 100 μM, final concentration in each well) and incubated for 3, 8, 24, 48, 72 and 144 h. In each plate, control cells were included and grown in the same conditions as the treated cells. After each exposure time, the plates were washed twice with sterile PBS (200 μl) and the surviving cells were quantified using the supravital stain neutral red assay [17]. For that, a stock solution of 1 mg/ml of neutral red dissolved in double-distilled water was prepared and diluted (1 + 1) just before use with 1.8% NaCl (working solution). A volume of 50 μl (working solution) was added to each well and incubated for 1.5 h. After removal, the cells were washed twice with phosphate-buffered saline. Finally, the dye was extracted from the cells by addition of 100 μl 0.05 M NaH_2PO_4 prepared in 50% of ethanol. Optical density was read at 540 nm using a microtite plate reader (Labsystems iEMS Reader MF).

In a previous experiment, a standard curve between different numbers of cells (5×10^3–60×10^3 cells/well) and the absorbance, was generated for this assay.

2.7. Cell cycle analysis

A density of 1×10^6 cells/well was seeded into 6-well culture plates. After 24 h, the plates were treated with serial concentrations of MP, NP or free cisplatin (2.5, 10 and 50 μM, final concentration in each well) and incubated for 10, 24, 48, 72 and 144 h. In each plate, control cells were included and grown in the same conditions as the treated cells. At each time-point, cells were detached by trypsine and washed twice with 2 mL of PBS. The pellets were incubated 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 samples were performed on a Becton–Dickinson FACScan flow cytometer using the CellQuest Software.

2.8. Determination of caspase-3 activity

Caspase 3 activity was determined using the Caspase-3/CPP32 colorimetric Assay Kit (BioVision, Inc., Palo Alto, CA, USA) following the experimental protocol used for cell cycle analysis. In brief, at each time-exposure attached and detached cells were isolated by trypsine and centrifuged at 240*g* for 10 min. The supernatants were removed and the pellets were resuspended in 55 μl of lysis buffer and incubated for 10 min at 4 °C followed by centrifugation at 20,000*g* for 10 min. Aliquots (50 μl) of supernatant were placed in a 96-well microplate containing reaction buffer. Substrate was added and the samples were incubated overnight at 37 °C. Activity was measured at 405 nm. A standard curve constructed with caspase-3 was also carried out to extrapolate the experimental data.

2.9. Statistical analysis

Particle diameters, ζ-potential, "in-vitro" release and "in-vitro" cytotoxicity studies were performed in triplicate. The data are represented as mean ± standard deviation (SD). Data were analyzed using the nonparametric Kruskal–Wallis test (for more than two groups) or the Mann–Whitney *U*-test (for two groups). Statistical significance was set at $P < 0.05$.

3. Results

3.1. Physico-chemical characterization

Although the method W/O/W seems to involve a relatively simple process, the characteristics of the final product may largely vary depending on the process variables. The particle size was influenced by the stirring speed. Two homogenizations with the Ultra-Turrax led to obtain particles with a higher diameter in comparison with the particles obtained by two homogenizations with the probe sonication, as shows the Table 1. The PLGA polymer conferred a negative ζ-potential in both formulations with an

Table 1
Characteristics of PLGA micro- and nanoparticles involved in the "in-vitro" studies

	Nanoparticles		Microparticles	
	A	B	A	B
Size (μm)	0.18 ± 0.02	0.21 ± 0.02	9.21 ± 3	8.87 ± 2
ζ-Potential (mV)	−18.7 ± 0.5	−18.3 ± 0.3	−22.1 ± 0.8	−23.1 ± 0.7
Loading (μg drug/mg polymer)	7.2 ± 1.5	–	9.2 ± 2.2	–
Drug concentration (μg drug/mL)	32.2 ± 6.6	–	41.1 ± 9.8	–

All results are expressed as the mean of three replicates. (A) Particles loaded with cisplatin; (B) particles without cisplatin.

average value of −22.13 and −18.73 mV for MP and NP, respectively. No significant differences were observed in the size and ζ-potential between loaded and non-loaded cisplatin formulations.

3.2. Morphological studies

Fig. 1 shows representative images of the external structure of the MP and NP, obtained by using scanning electron microscopy (SEM). MP and NP populations were spherical with a diameter similar than obtained by dynamic light scattering. The appearance of MP formulated with and without cisplatin was indistinguishable. Similar results were observed for NP population.

3.3. Encapsulation efficiency and "in-vitro" cisplatin release from particles

Table 1 shows that the encapsulation of cisplatin, expressed as concentration, was higher ($P < 0.01$) in the MP than in NP, as expected. However, the loading or the encapsulation efficiency was similar for both formulations: 11.23% and 10.33% (w/w) for NP and MP, respectively. This encapsulation efficiency was calculated as the amount of cisplatin recovered from each particle relative to the initial amount of cisplatin used for the fabrication process. The loading obtained for NP and MP without prior saturation of the external phase was extremely low (∼1%). In order to know which could be the best condition of saturation to reach a significant degree of encapsulation, different amounts of cisplatin in the external and internal aqueous phase, were tested. These results, showed in Fig. 2, indicated that within the range of cisplatin concentrations investigated, 1 mg/mL and 2.5 mg (∼1.7 mg/mL, maximum Tris- solubility of cisplatin) in the external and in the internal aqueous phase, respectively, represented the most favourable condition for formulating the particles, although no statistical ($P > 0.05$) difference was found regarding to a concentration of 2 mg/mL in the external phase. In the case of NP, similar results (1 mg/mL) were found for the external phase, while for the internal a concentration of cisplatin of 2.5 mg/mL (the maximum solubility in water) was used.

The "in-vitro" cumulative release profile of cisplatin measured by HPLC and expressed as concentration, is represented in Fig. 3. The analytical assay was sensible enough to measure cisplatin in a wide range of concentrations with a limit of detection of 0.2 ng/mL. In each experiment, a calibration curve was constructed with known cisplatin amounts. The inter- and intra-assay precision was <5%.

The release kinetics of cisplatin from NP showed a biphasic profile, characterized by a rapid initial burst effect followed by an exponential phase and reaching a plateau after 30 days. However, a sigmoidal shape reflecting four phases can be observed for MP: an initial burst during the first day of release, followed by a lag-time period of approximately 5 days, then a constant release period for 25 days, and finally a phase of very slow release reaching a plateau over 5 days (40–45 days of release study). The burst effect for the two formulations was very different,

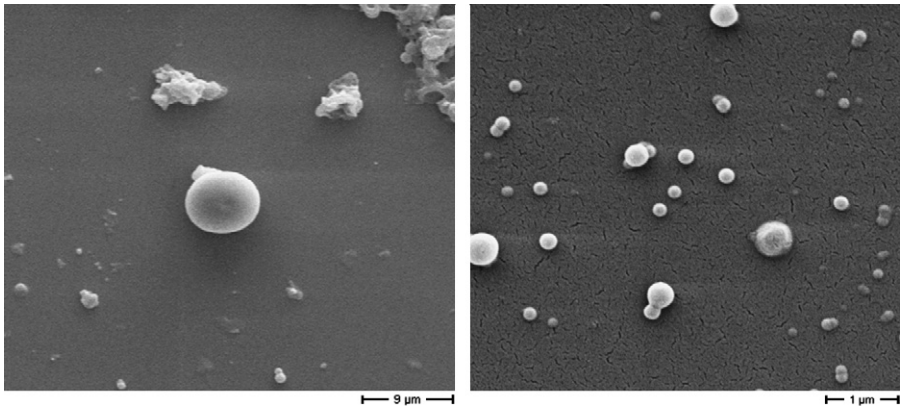

Fig. 1. Scanning electron micrographs of PLGA particles prepared with W/O/W method using different protocols. Left panel shows the MP and right panel NP.

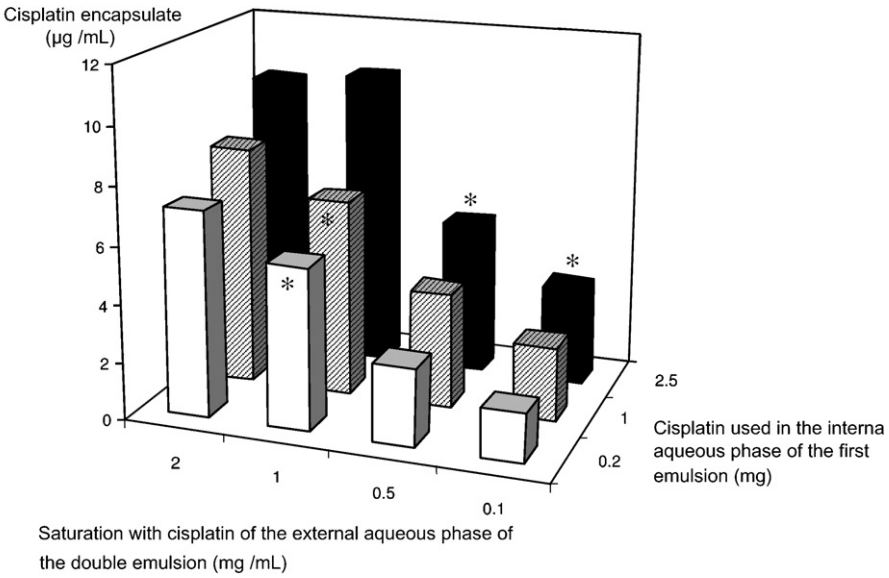

Fig. 2. Cisplatin encapsulated using different conditions for the external and internal aqueous phases, respectively, during the development of MP. Statistical differences were calculated in relation to the selected conditions ($^{*}P < 0.05$).

20% for MP vs. 50% for NP, during the first day of the study. The final concentration reached after one month, was accepted as the encapsulated cisplatin concentration. The differences represented in Fig. 3 suggest that cisplatin entrapment in the MP could significantly retard its "in-vitro" release and consequently lead to a more constant effect over time.

3.4. Cytotoxicity

To evaluate the antiproliferative effect of the unloaded formulations, different amounts of NP and MP (0.25, 0.5, 0.75 and 1 mg) were tested in microplates seeded with different densities of cells (5, 10 and 20×10^3 cells/well). Within the evaluated range for cells and quantities of particles, the cell growth curves of control (without particles) and treated groups were similar, supporting the idea that these systems are not toxic themselves (data not shown). Based on data, a maximum amount of 1 mg of particles/well and a density of 20×10^3 cells/well were the selected conditions to evaluate the effect of cisplatin encapsulated, and in solution.

The cytotoxic effect, characterized for different concentrations of free and encapsulated cisplatin after different time exposures, is represented in Fig. 4. Treatments of DHDK12-Prob with cisplatin did not change the profiles in the cell growth curves in the first 8 h. However, after 24 h a concentration-dependent cytotoxicity, with a statistical difference ($P < 0.05$), was found for encapsulated *vs.* free cisplatin. This difference was also observed between MP and NP, supporting the fact that MP seems to be the less toxic formulation, as was expected in relation to the profile found in the "in-vitro" release study. In fact, MP showed statistical differences compared to NP at 24 and 48 h. Nevertheless, at 72 and 144 h the statistical difference between formulations disappeared and was only found in relation to the group treated with cisplatin in solution.Therefore, MP and NP displayed the same behaviour for all doses after 72 h of each treatment. These results expressed as values of the IC_{50} [the concentration of cisplatin that is able to inhibit the 50% of the maximum antiproliferative effect at each time] showed that at 24 h this value was reached only for free cisplatin (18 µM) and in the borderline for NP (100 µM). This large difference for IC_{50}, was dramatically reduced at 48 h and especially at 72 h of treatments, as shows Fig. 5, suggesting the presence of two effects, one cell-dependent and the other one time-dependent for polymer degradation. Both effects seem to be responsible for the decrease observed in the antiproliferative effect of the formulations. The control groups for the three experiments displayed the same behaviour since any statistical difference was observed between them. In this case, the growth of control cells was expressed as the number of survival cells because with these curves, it was demonstrated that the experimental conditions in the three situations were the same. The coefficient of variation (%) ranged between 8–10% through the time course of three control groups.

3.5. Cell cycle analysis

To investigate the differences in the reduction of the antiproliferative effect of free cisplatin, NP and MP, an analysis of the cell cycle after different treatments (2.5, 10 and 50 µM) was carried out. Fig. 6 shows that all treatments in general, lead to a time-dependent reduction in the percentage of cells in phase G0/G1. However, this reduction was more significant for free cisplatin treatments. In addition, the percentage of cells in phase

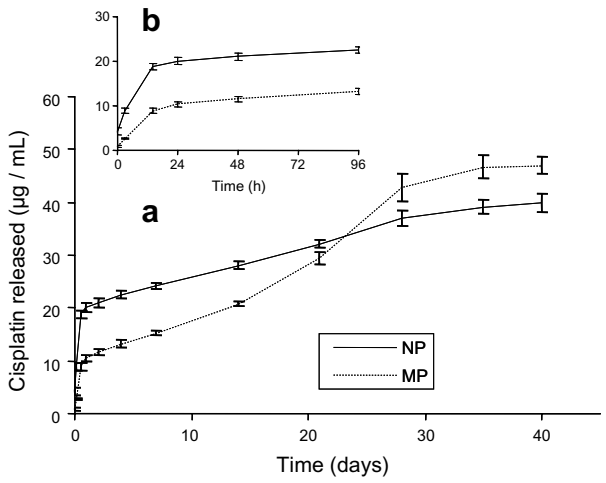

Fig. 3. "In-vitro" release profiles of cisplatin, expressed in concentration (μg/mL), from NP (dashed lines) and from MP (solid line), (a) Data represent the mean ± SD ($n = 3$). In the small graph is represented in detail, the first times of these profiles, (b).

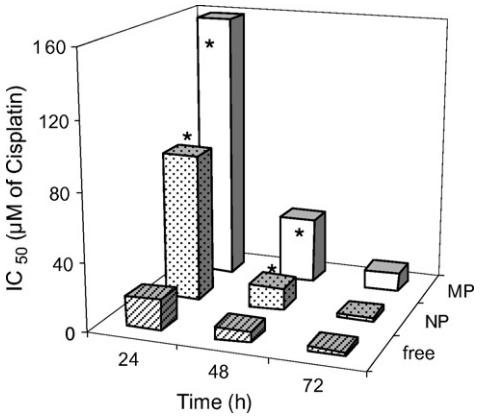

Fig. 5. Time course of the IC_{50} for cisplatin in solution compared to cisplatin encapsulated. Data represent the mean ± SD ($n = 3$) ($^{*}P < 0.05$ in relation to the free cisplatin treatments).

G2/M was increased for all NP and MP treatments reaching a plateau between 24 and 48 h. This profile was also observed for concentrations of free cisplatin lower than 10 μM, whereas for higher concentrations such as 50 μM, a rapid reduction of cells in this phase was evident. In this case, the loss of cells from the G0/G1 and G2/M phases, was accompanied by the appearance of a Sub-G0/G1 peak (Fig. 7). Therefore, on the basis of these results, low doses of free cisplatin induced similar effects than cisplatin released from both formulations at higher doses 50 μM, supporting the idea that a dual mechanism dose-dependent could be involved, in part, in the resistance process of this agent.

3.6. Caspase 3 activation

Fig. 8 shows the profiles corresponding to the activity of caspase-3 for each of the different treatments with free and encapsulated cisplatin. The activity of this protein began to increase at 10 h of the each treatment reaching a peak at different times depending on the dose. The maximum induced peaks for free cisplatin were observed at 72 h for lower doses (2.5 and 10 μM), and at 24 h for higher

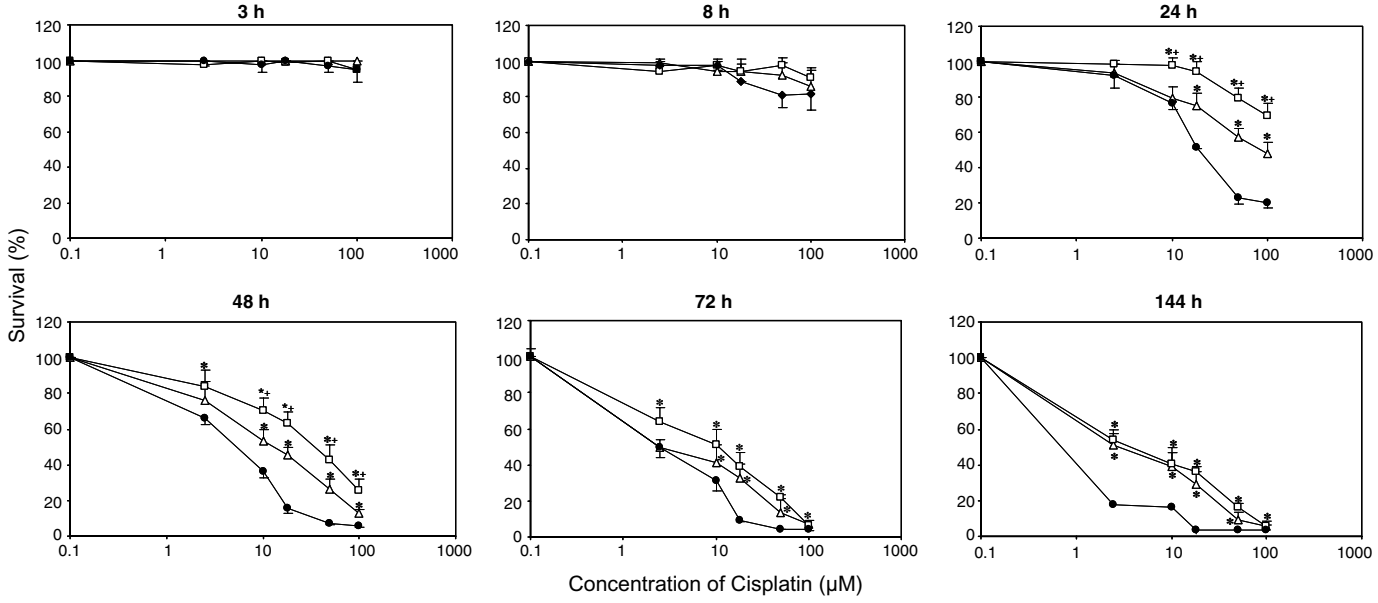

Fig. 4. Effect of different doses and exposure times of cisplatin in solution or encapsulated on the cell-proliferation. This effect is expressed as the percentage of survival cells at each point in relation to the control group (not treated). Data represents the ± SD ($n = 3$) and each panel a different time of exposure. Free cisplatin treatments (●); NP (Δ) and MP (□). ($^{*}P < 0.05$ in relation to the free cisplatin treatments; $^{+}P < 0.05$ in relation to the NP treatments).

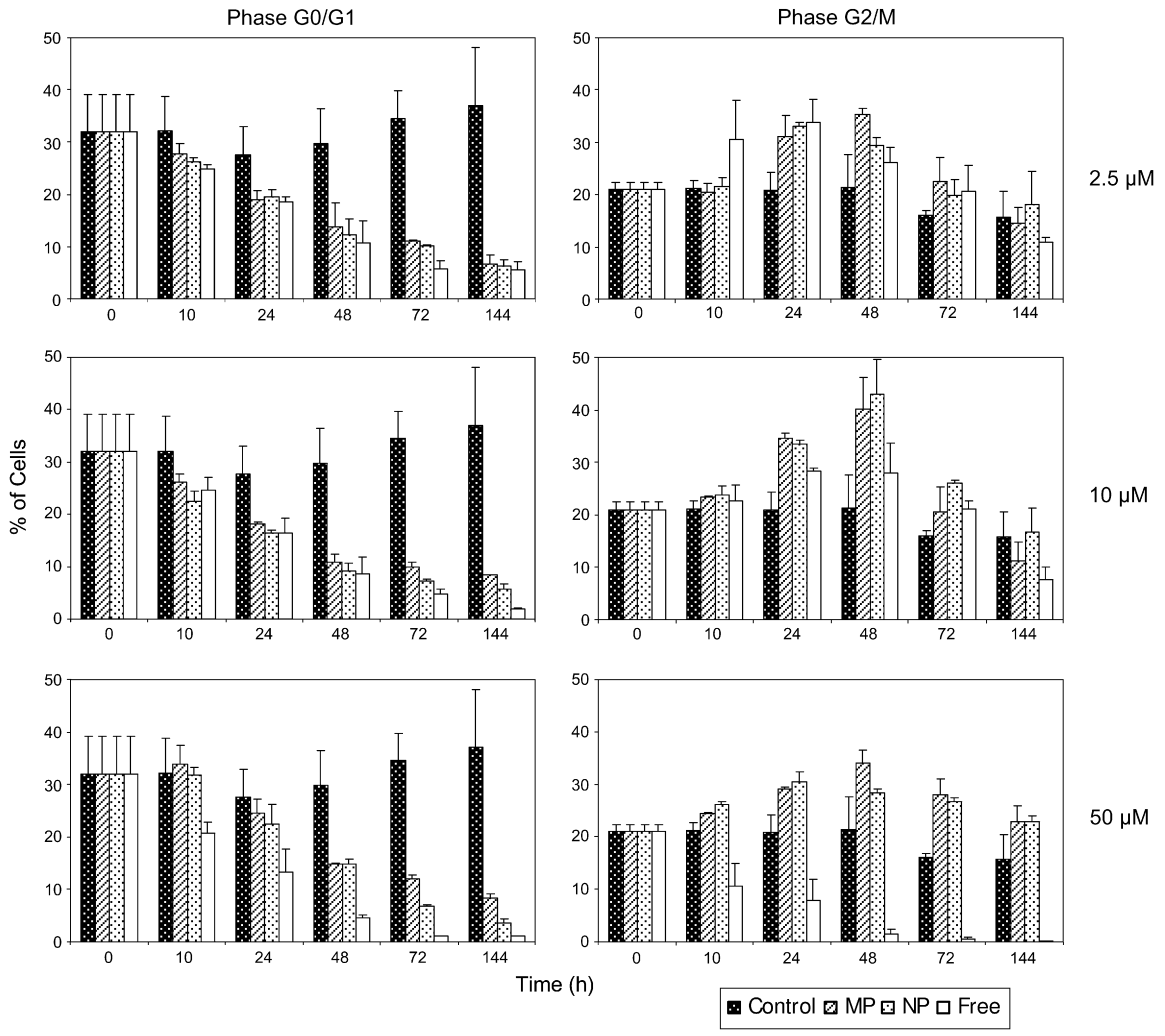

Fig. 6. Cell-cycle distribution observed in cells exposured to different time-points and doses (2.5, 10 and 50 μM) of free- and encapsulated cisplatin. Data represent the mean ± SD ($n = 3$).

(50 μM). However in the case of the treatments with MP, all peaks were displaced at 72 h for the low doses and, at 48 h for high, 50 μM. NP displayed similar results than those obtained for MP. An influence of the dose on the time needed to reach the maximum activation, and then the apoptosis can be concluded.

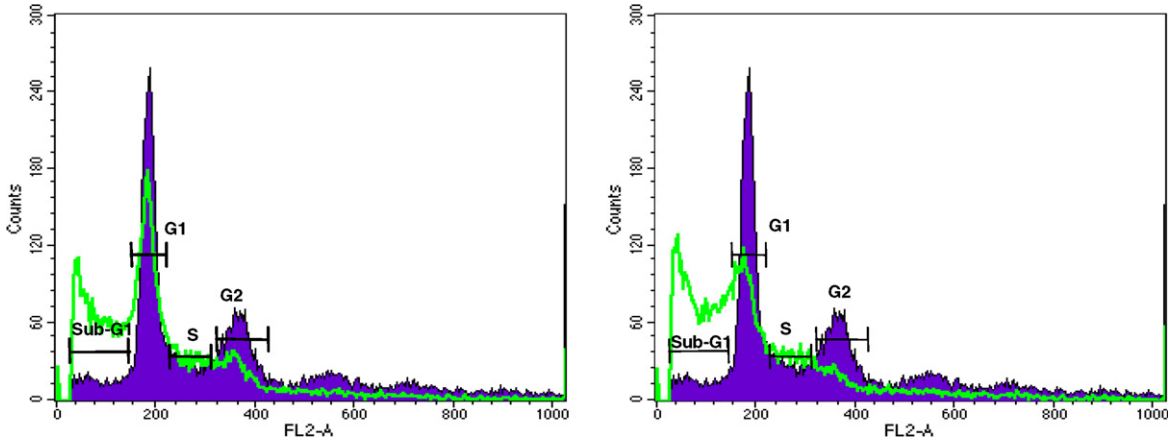

Fig. 7. Cell cycle distribution after 12 and 24 h of treatment with 50 μM of free cisplatin. The loss of cells in G0/G1 and G2/M is attributable to the increase in the Sub-G1 population.

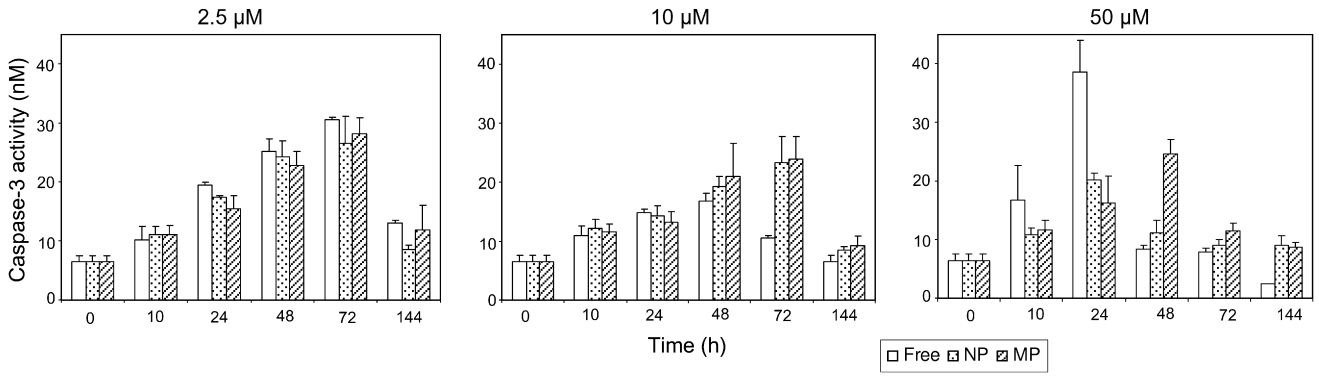

Fig. 8. Time course of caspase-3 activated in each of the used treatments for the analysis of the cell-cycle. The activity of caspase-3 was expressed in relation to the standard curve carried out with commercial protein. Data represent the mean ± SD ($n = 3$).

4. Discussion

In this study, we have investigated the feasibility of the W/O/W method to encapsulate cisplatin in PLGA polymeric systems. Two protocols were developed to study the influence of some process variables in the particle size, the loading efficiency, the "in-vitro" release profile from these systems and consequently the cytotoxic effect of cisplatin caused in each situation.

Although many methods and protocols are described in the literature to develop microspheres of cisplatin [2,8,9] not many concern nanoparticles [1]. For this reason, we decided to develop both formulations, MP and NP, and test them in culture cells evaluating the possible advantages for each formulation.

The initial results showed that the differences in the size observed in Table 1 were dependent on the mixing speed of the homogenizer used to prepare the formulations. Two stirring with the Ultra-turrax system instead of the probe sonication led to a higher particle size in the formulation. However, it could be observed that the size was really dependent on the characteristics of the second homogenization. In fact, Díez and Tros de Ilarduya [11], found that the use of probe sonication for the first emulsion followed by the Ultra-turrax system at different times and potencies led to obtain particles with a different diameter but within a narrow range, from 3.4 to 5.7 μm. On the other hand, the cisplatin content was higher for MP than for NP. This result was expected because it is well known that this parameter always depends on the particle size. However, the encapsulation efficiency for both, MP and NP, presented similar values [11.23% vs. 10.33% (w/w)], suggesting that the solvent evaporation method was responsible for this loading and more or less independent of the protocols. The low encapsulation efficiency is very common in cisplatin-microparticles. The main reason for that is the physico-chemical characteristics of cisplatin [1,2,8]. In fact, cisplatin is practically insoluble in organic solvents and slightly soluble in water, which means that it is very difficult to manufacture polymeric sustained-release systems that can maintain adequate concentration for long periods of time (>2 weeks). This characteristic together with the use of the solvent evaporation method during the preparation of formulations, led to the presence of a significant initial burst effect [2]. Nevertheless, in this work NP and MP were formulated under this method, with the only difference in the solvents used in the first emulsion, water with dichloromethane for NP and Tris–HCl (pH 7.4) with chloroform for MP, founding a difference in the burst effect of 50% vs. 20% for NP and MP, respectively, on the first day. This difference in the burst effect could be explained by the relationship between the specific surface area and the size of each formulation. Therefore, a higher burst effect is expected for NP than for MP.

On the other hand, and in order to obtain a good level of drug in the particles, we tested two different copolymers, observing that the use of the RG502H in comparison with RG752 led us to obtain much higher loadings (data not shown). Similar results have also been reported for DNA encapsulation in microparticles with these Resomers and under the same solvent evaporation method [11].

The study about the saturation of external aqueous phase exhibited an increase in the loading of the drug reaching a plateau between 1 and 2 mg/mL of cisplatin. A significant influence was also evidenced by the increase of the concentration of cisplatin in the internal aqueous phase, as is represented in Fig. 2. This kind of study has not been carried out before, although the saturation of the external aqueous phase for increasing the loading of cisplatin has been previously reported by several authors using different conditions [1,8].

The amount of cisplatin released in the "in-vitro" study was measured by a HPLC technique, which was developed following similar assays previously described by other authors [15,16]. It was interesting to note that the profiles found in this part of the work showed a correlation with the results about the antiproliferative effect and apoptosis obtained in a posterior experiments carried out with culture cells (Figs. 4 and 8).

The antiproliferative effect caused by the different treatments with free and encapsulated cisplatin was clearly evidenced at 24 h of the treatments, whereas until that point

no differences from the control group could be observed. The change of the IC_{50} (Figs. 4 and 5) for NP and MP supports the view that the cytotoxicity was reduced for the formulations. The statistical difference in the degree of cytotoxicity found between MP and NP at 24 and 48 h suggested that the rapid release of cisplatin from NP led to an increase in the death cells compared to the effect of MP. In addition, the reduction of the difference in IC_{50} for the three groups through the time of the experiment observed in Fig. 5, could be explained by the fact that the particles might provide a matrix structure increasing the surface area to promote cell adhesion and their proliferation and growth. A possible consequence of this process, would be an acceleration in the degradation of the polymer, especially for MP, modifying its "in-vitro" release profile. This mechanism of adhesion appears to be, in part, associated with the presence of PVA in the structure of the formulations. The hydroxyl groups of PVA seem to play a positive role in interacting with the polar groups on the cell surface providing an adhesion of the cells in the polymeric structures [3].

On the other hand, is very well known that the cytotoxic effect of platinum derivatives is due to DNA damage resulting in a cell cycle arrest, which occurs mainly at the G2/M transition [18]. This effect was more evident for the formulations than for free cisplatin, as is shown in Fig. 6. The behaviour of all doses (2.5–50 μM) of cisplatin encapsulated was similar to low doses (2.5 and 10 μM) of free cisplatin, resulting in a G2/M arrest of the cell cycle. However for high doses such as 50 μM, a very rapid loss of cells from G2/M phase (12 h of treatment) was accompanied by an increase in the sub-G0/G1 (see Fig. 7). These results were consistent with those found by Cumming and Schnellmann [19], where the authors attributed to apoptosis in renal proximal tubular cells caused by high doses of cisplatin (50 μM) the renal dysfunction found in patients. In addition, continuous treatments with low doses of cisplatin (0. 125 μg/mL) were more effective in the formation of DNA-adducts than a single high dose (1 μg/mL), which induced very quick accumulations of cells in sub-G1. This accumulation tended to decrease as the time of exposure to cisplatin increased [6].

Cisplatin induced apoptosis through caspase signalling pathway. One of the effectors implicated is the caspase-3, which plays a significant role in the apoptosis caused by cisplatin [19–21]. In this work, a repression of cell cycle related to an arrest in G2/M phase of the cells treated with cisplatin led to an activation of caspase-3. This activation showed different profiles for free- and encapsulated cisplatin depending on the exposure time and the dose. In general, treatments with high doses showed a rapid activation/deactivation of this protein due to the cell death induced by them (Figs. 4, 6 and 8). For example, 50 μM of free cisplatin, proved extremely toxic since after 24 h of treatment, only a 20% of cells survived. In addition, at this time, a cascade of events was found: a significant loss of cells in G2/M phase followed by an increase of the sub-G1 population and the presence of a high peak of activated capase-3. However, for both formulations and low doses of free cisplatin, a more gradual and continuous activation of caspase-3 led to more gradual death cell. Therefore, these data seem to support the idea that sustained release formulation of cisplatin could increase the efficacy of the treatments by decreasing the adverse effects. On the basis of these results, the next idea for both formulations will be to explore in "in-vivo" their possible use in colon cancer by using different routes of administration: intraperitoneally for MP [22] and intravenously for NP.

In conclusion, the versatility of the W/O/W method allowed, with a very good efficiency, the encapsulation of cisplatin in PLGA particles with different characteristics depending on the protocol used for their manufacture. The cytotoxic effect caused by encapsulated cisplatin in both formulations was lower than for the free-agent at most of the doses and exposure times used in this work. A more effective repression of the checkpoints of the cell cycle was also found for both formulations leading to an accumulation of cells in G2/M phase. The induction of G2/M arrest induced a gradual activation of caspase-3, a protein directly implicated in the cisplatin-apoptosis. Therefore, the dose and the exposure time were two essential parameters for cisplatin to induce effective apoptosis. All these data together seem to support the use of sustained-release formulations to increase the efficacy of this agent while decreasing the adverse effects.

Acknowledgements

The authors thank Dr. García-Olmo for generously providing DHD-K12ProB cells. This work was financially supported by the University of Navarra Foundation, the Government of Navarra and the Roviralta Foundation. Daniel Moreno was supported by a grant from the Government of Navarra.

References

[1] K. Avgustakis, A. Beletsi, P. Panagi, A.G. Karydas, D.S. Ithakissios, PLGA-mPEG nanoparticles of cisplatin: "in-vitro" nanoparticle degradation, "in-vitro" drug release and "in-vivo" drug resistance in blood properties, J. Control. Release 79 (2002) 123–135.

[2] J. Fujiyama, Y. Nakase, K. Osaki, C.H. Sakakura, H. Ymagishi, A. Hagiwara, Cisplatin incorporated in microspheres: development and fundamental studies for its clinica application, J. Control. Release 89 (2003) 397–408.

[3] S.K. Sahoo, A.K. Pnada, V. Labhasetwar, Characterizacion of porous PLGA/PLA microparticels as a scaffold for three dimensional growth of breast cancer cells, Biomacromolecules 6 (2005) 1132–1139.

[4] M. Kartalou, J.M. Essigmann, Mechanisms of resistance to cisplatin, Mutat. Res. 478 (2001) 23–43.

[5] X. Yan, R.A. Gemeinhart, Cisplatin delivery from poly(acrylic acid-co-methyl methacrylate) microparticles, J. Control. Release 106 (1-2) (2005) 198–208.

[6] S. Kishimoto, Y. Kawazoe, M. Ikeno, S. Fukushima, Y. Takeuchi, Continuous exposure to low-dose cisplatin and apoptosis, Biol. Pharm. Bull. 28 (2005) 1954–1957.

[7] T. Boulikas, G.P. Stathopoulos, N. Volakakis, M. Vougiouka, Systemic lipoplatin infusion results in preferential tumor uptake in human studies, Anticancer Res. 25 (2005) 3031–3039.
[8] L. García-Contreras, K. Abu-Izza, R.D. Lu RD, Biodegradable microspheres for direct brain injection: preparation and characterization, Pharm. Dev. Technol. 2 (1) (1997) 53–65.
[9] T. Tamura, F. Fujita, M. Tanimoto, M. Koike, A. Suzuki, M. Fujita, Y. Horikiru, Y. Sakamoto, T. Suzuki, H. Yoshino, Antitumor effect of intraperitonela administration of cisplatin-loaded microspheres to human tumor xenografed nude mice, J. Control. Release 80 (2002) 295–307.
[10] R.K. Gupta, M. Singh, D.T. O'Hagan, Poly(lactide-co-glycolide) microparticles for the development of single-dose controlled-release vaccine, Adv. Drug Deliv. Rev. 32 (1998) 225–246.
[11] S. Díez, C. Tros de Ilarduya, Versatility of biodegradable poly(D,L-lactic-co-glycolic acid) microspheres for plasmid DNA delivery, Eur. J. Pharm. Biopharm. 63 (2006) 188–197.
[12] R. Verrijk, I.J. Smolders, N. Bosnie, A.C. Begg, Reduction of systemic exposure and toxicity of cisplatin by encapsulation in poly-lactide-co-glycolide, Cancer Res. 52 (23) (1992) 6653–6656.
[13] K. Itoi, C.Y. Tabata, O. Ike, Y. Shimizu, M. Kuwara, M. Kyo, S.H. Hyon, Y. Ikada, "In-vivo" suppressive effects of copoly(glycolic/L-lactic acid) microspheres containing CDDP on murine tumor cells, J. Control. Release 42 (1996) 175–184.
[14] J.M. Koziara, T.R. Whisman, M.T. Tseng, J.R. Mumper, "In-vivo" efficacy of novel paclitaxel nanoparticles in paclitaxel-resistant human colorectal tumors, J. Control. Release 112 (2006) 312–319.
[15] V. Augey, M. Cociglio, M. Galtier, R. Yearoo, V. Pinsani, F. Bressolle, High-performance liquid chromatographic determination of *cis*-dichlorodiammineplatinum (II) in plasma ultrafiltrate, J. Pharm. Biomed. Anal. 13 (1995) 1173–1178.
[16] A. López-Flores, R. Jurado, P. García-López, A high-performance liquid chromatographic assay for determination of cisplatin in plasma, cancer cells, and tumor samples, J. Pharmacol. Toxicol. Methods 52 (3) (2005) 366–372.
[17] C.W.G.M. Löwik, M.J. Alblas, M. van de Ruit, S.E. Papapoulos, G. van der Pluijm, Quantification of adherent and nonadherent cells cultured in 96-well plates using the supravital stain neutral red, Anal. Biochem. 213 (1993) 426–433.
[18] C. Voland, A. Bord, A. Péleraux, G. Pénarier, D. Carrière, S. GAliègue, E. Cvitkovic, O. Jbilo, P. Casellas, Repression of cell cycle related protein by oxaliplatin but not cisplatin in human colon cancer cells, Mol. Cancer Ther. 5 (2006) 2149–2157.
[19] B.S. Cummings, R.G. Schnellmann, Cisplatin-induced renal cell apoptosis: caspase 3-dependent and -independent pathways, J. Pharmcol. Exp. Ther. 302 (2002) 8–17.
[20] Y.J. Wu, L.L. Muldoon, E.A. Neuwelt, The chemoprotective agent *N*-acetylcysteine blocks cisplatin-induced apoptosis through caspase signalling pathway, J. Pharmacol. Exp. Ther. 312 (2005) 424–431.
[21] D.T. Dickey, Y.J. Wu, L.L. Muldoon, E.A. Neuwelt, Protection against cisplatin-induced toxicities by *N*-acetylcysteine and sodium thiosulfate as assessed at the molecular, cellular and "in-vivo" levels, J. Pharmacol. Exp. Ther. 314 (2005) 1052–1058.
[22] K. Narita, H. Ikuta, Y. Hamabe, Y. Kuroda, Experimental study on paratumoral injection of cisplatin-loaded microspheres for gastric cancer, J. Med. Sci. 45 (1999) 229–243.