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ORIGINAL ARTICLE

In vivo comparative study of the cytotoxicity of a liposomal formulation of cisplatin (lipoplatin™)

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Abstract
Purpose Cisplatin is one of the most effective cytotoxic agents in the treatment of solid malignancies, but its use is limited by several side effects. Among them, peripheral neurotoxicity can be dose limiting. A liposomal formulation of cisplatin, Lipoplatin™, was developed to reduce the systemic toxicity of cisplatin but without preventing its efficacy. The aim of this study was to use an animal model to establish, through a multimodal approach, whether chronic treatment with two different schedules of Lipoplatin™, selected within the range of its anticancer effective dose, is less neurotoxic than cisplatin administration.
Methods Female Wistar rats were treated intraperitoneally with cisplatin at a dose of 4 mg/kg or with Lipoplatin™ at doses delivering 12 or 24 mg/kg of cisplatin once weekly for 4 weeks. General toxicity was assessed by daily observation, body weight change, hematological and blood chemistry analysis, and histopathology of liver and kidney. The onset of peripheral neurotoxicity was assessed by measuring tail nerve conduction velocity (NCV), morphological and morphometric analysis of dorsal root ganglia (DRG), and morphological analysis of the sciatic nerve.
Results Cisplatin induced a statistically significant reduction in body weight, the development of renal failure, and impairment in NCV with pathological alterations in the DRG and sciatic nerve. By contrast, Lipoplatin™ was markedly less nephrotoxic, and no significant weight gain reduction was observed in animals treated with both doses of the drug. Moreover, the lowest dose induced less severe damage to the peripheral nervous system with a moderate decrease in NCV and mild pathological alterations in DRG and the sciatic nerve.
Conclusions The results suggest that Lipoplatin™ 12 mg/kg is less neurotoxic than cisplatin 4 mg/kg, thus opening up the possibility of using this new formulation in future studies where its anticancer activity and the peripheral neurotoxicity will be assessed in parallel.

Keywords Cisplatin · Liposomes · Lipoplatin™ · Peripheral neurotoxicity · Wistar rats · Nerve conduction velocity

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Introduction

Chemotherapy, radiotherapy, and surgery continue to be the mainstay treatments of cancer, although targeted therapies are becoming increasingly important. Cisplatin (CDDP), cis-diamminedichloroplatinum (II), since its discovery in 1965 by Rosenberg, identification in 1969 and first clinical application in the early '70s, continues to be a cornerstone in modern anticancer chemotherapy [1–3]. CDDP is largely used for the treatment of solid tumors such as ovarian, testicular, and bladder cancers [4–6], as well as

for first-line chemotherapy against cancer of the head-and-neck, lung, stomach, esophagus, colon, uterus, cervix, and prostate [7–13]. Since the first attempts to use CDDP, its clinical use has been limited by several severe side effects, including principally nephrotoxicity [14] and peripheral neurotoxicity [15], followed by ototoxicity [16], gastrointestinal toxicity, and asthenia. Since a reduction in renal damage has successfully been achieved with hydration and a reduction in the gastrointestinal side effects with antiemetics, peripheral neurotoxicity has become the most important adverse effect associated with CDDP chemotherapy. Its symptoms and signs include numbness, tingling, paresthesias in the extremities, ataxia with difficulty in standing and walking, decreased vibration sense, and loss of deep tendon reflexes. Higher platinum concentrations in the peripheral than in the central nervous system seem to correlate with the clinical symptoms and severity of peripheral neurotoxicity [17–19]. In the last few years, nanotechnology has opened up new horizons in molecular oncology making it possible to reformulate new and preexisting drugs into nanoparticles with altered biodistribution and improved pharmacokinetics with the aim of reducing side effects and of enhancing therapeutic efficacy by targeting the tumors. Lipoplatin™ is a formulation of CDDP wrapped up in tumor targeted 110-nm liposome nanoparticles [20]. Published data have evidenced reduced nephrotoxicity of Lipoplatin™ in comparison with CDDP, while only limited information is available about the effect of chronic administration of Lipoplatin™ on the peripheral nervous system [21]. For this reason, in the present study, we evaluated, through a multimodal approach, the peripheral neurotoxicity induced by chronic administration of two different doses of Lipoplatin™ in a rat model and we compared the results with those obtained in a well-established CDDP model.

Materials and methods

Animal husbandry

Thirty-two female Wistar rats (180–200 g on arrival at the housing room, Harlan Italy, Correzzana, Italy) were used for the experiment. The care and husbandry of the animals were in conformity with the institutional guidelines in compliance with national (D.L. n. 116, *Gazzetta Ufficiale della Repubblica Italiana*, suppl. 40, February 18, 1992) and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, December 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996). The experimental plan was preliminarily examined and approved by the *ad hoc* committee of the University of Milano-Bicocca. Animals were randomized and housed two per cage in a limited access animal facility where room temperature and relative humidity were set at $22 \pm 2°C$ and $55 \pm 10\%$, respectively. Artificial lighting provided a 24-h cycle of 12 h light/12 h dark (7 am–7 pm).

Experimental design

At the beginning of the study, rats were randomly divided into 4 groups: untreated controls (CTRL; $n = 8$), cisplatin 4 mg/kg (CDDP 4; $n = 8$), Lipoplatin™ 12 mg/kg equivalent to CDDP 12 mg/kg (LIPO 12; $n = 8$), and Lipoplatin™ 24 mg/kg equivalent to CDDP 24 mg/kg (LIPO 24; $n = 8$). CDDP and Lipoplatin™ were administered once weekly for 4 weeks. To assess the development of peripheral neurotoxicity, nerve conduction velocity (NCV) was evaluated after 3 administrations and at the end of the study. At the end of the treatment period, animals were killed by CO_2 inhalation followed by cervical dislocation, and sciatic nerves, L4–L5 dorsal root ganglia (DRG), liver, and kidney were collected from all animals for subsequent analysis. While killing, blood samples were collected from all the animals.

Drugs

CDDP and its liposomal formulation Lipoplatin™ (provided by Regulon AE, Athens, Greece) were administered intraperitoneally. CDDP was dissolved in sterile saline solution immediately before each administration, while Lipoplatin™ was provided in 50-ml vials at a concentration of 3 mg/ml ready for use. Drug doses were adjusted to individual animal weights at each administration.

General toxicity

General toxicity was monitored by daily observation, and body weight changes were measured once weekly. Hematological and blood chemistry analysis as well as histopathology of the liver and kidney were performed on the specimens collected at the end of study.

Hematological and blood chemistry determinations

At the end of treatment, whole blood was obtained from all animals through abdominal aorta puncture and collected in a heparinated tube for complete blood cell determination performed with a PENTRA 60 C+ device (Horiba ABX Diagnostic Montpellier, France). Serum was obtained by the centrifugation of clotted blood at 2,500*g* for 15 min at 4°C and used for urea, creatinine, AST, and ALT determination with an automatic MIRA PLUS system (Horiba ABX Diagnostic Montpellier, France) [22].

Histopathology of kidney and liver

At the moment of killing the animal, kidney and liver specimens were fixed in 10% formalin for 7 days, and after fixation, the samples were paraffin embedded. Transverse sections (4 μm thick) were obtained, stained with hematoxylin and eosin, and observed with a Nikon Coolscope light microscope (Nikon Instruments, Calenzano, Italy).

Determination of tissue cisplatin concentration

The tissue total platinum concentration was determined on frozen sciatic nerve, and DRG, kidney, and liver specimens collected from 3 animals/group killed 24 h after the last administration of the drugs. For each tissue, a calibration with control standard tissue was generated. All frozen test samples and standards were treated for a digestion process with a specific HNO_3: HCl solution (1:3). The samples obtained after digestion were analyzed by "Atomic Absorption" (Analyst 600 Perkin Elmer, Monza, Italy), and platinum tissue concentration was calculated accordingly.

Neurotoxicity

Neurophysiological assessment

NCV was determined in the tail nerve of each animal as previously described after 3 and 4 administrations [23–25]. The NCV in the tail nerve was assessed by placing recording ring electrodes distally in the tail, while the stimulating ring electrodes were placed 10 cm and 5 cm proximally with respect to the recording point. The latency of the potentials recorded at the 2 spikes after stimulation was registered (peak-to-peak), and NCV was calculated accordingly. All the neurophysiological were determined under standard conditions in a temperature-controlled room and with animal rectal temperature monitoring.

Histopathological examination of the sciatic nerve

The left sciatic nerves were removed, fixed by immersion in 3% glutaraldehyde, postfixed in OsO_4, embedded in epoxy resin, and used for light observations [23, 24]. Semithin sections were prepared from at least two tissue blocks for each animal. The sections were stained with toluidine blue and examined with a Nikon Coolscope light microscope (Nikon Instruments, Calenzano, Italy).

DRG histopathological and morphometric examinations

DRG of control and treated rats was used for histopathological and morphometric examinations. On toluidine blue–stained 1 μm thick semithin sections, DRG were analyzed with a computer-assisted image analyzer (ImageJ NIH software). The somatic, nuclear, and nucleolar sizes of DRG sensory neurons were measured in randomly selected sections according to previously reported methods on at least 300 DRG neurons/rat [26].

Statistical evaluation

The differences between all experimental groups in body weight, NCV, DRG morphometry, and tissue CDDP concentration were statistically evaluated using analysis of variance (ANOVA) and the Tukey–Kramer post-test (significance level set at $P < 0.05$).

Results

General toxicity

No mortality was observed during the treatment period among the animals treated with CDDP or Lipoplatin™ at different doses. No evident difference in animal behaviour between the animals treated with both doses of Lipoplatin™ and CTRL ones was observed while the CDDP 4-treated group showed piloerection and kyphosis from the first week of treatment.

Body weight changes

At the end of the study, animals treated with CDDP had a marked and statistically significant weight loss versus CTRL rats (mean value ± SD = 212.5 ± 9.35 g in CTRL animals versus 151.16 ± 20.03 g in CDDP 4-treated group, $P < 0.001$) that was already present after three drug administrations. In the same way, the treatment with CDDP induced a significant reduction in body weight also respect both doses of Lipoplatin™ (mean value ± SD = 151.16 ± 20.03 g in CDDP 4-treated rats versus 212.15 ± 7.25 g in LIPO12 group and 192.47 ± 9.37 g in LIPO 24-treated animals, $P < 0.001$). By contrast, no statistically significant weight gain reduction was observed in the animals treated with both doses of Lipoplatin™ versus CTRL rats.

Hematological and blood chemistry determinations

As shown in Table 1, CDDP administration induced kidney functional impairment, while urea and creatinine levels were similar in CTRL- and Lipoplatin™-treated rats.

Histopathology of kidney and liver

CDDP induced from moderate to marked nephropathy with evident tubular toxic changes mainly involving the outer

Table 1 Blood chemistry and hematological analysis at the end of the treatment period

Blood chemistry	Urea (mg/dl)	Creatinine (mg/dl)	ALT (UI/l)	AST (UI/l)
CTRL	35–53	0.36–0.42	41–59	63–70
CDDP 4	149–359	0.81–1.33	30–39	71–135
LIPO 12	34–50	0.36–0.43	41–75	96–119
LIPO 24	34–56	0.32–0.6	39–74	79–147

Hematology	RBC ($\times 10^6$/mm^3)	WBC ($\times 10^6$/mm^3)	PLT ($\times 10^3$/mm^3)	Hb (g/dl)	Hematocrit (%)
CTRL	7.42–7.88	4.4–8.5	648–804	14.7–15	42.3–43.2
CDDP 4	5.98–7.83	2.2–4.6	206–739	12.1–15.6	33.2–42.4
LIPO 12	6.83–7.96	5.5–8.7	655–769	13.6–15.4	39.3–44.7
LIPO 24	7.1–8.22	4.2–9.2	758–917	13.7–15.3	37.9–40.5

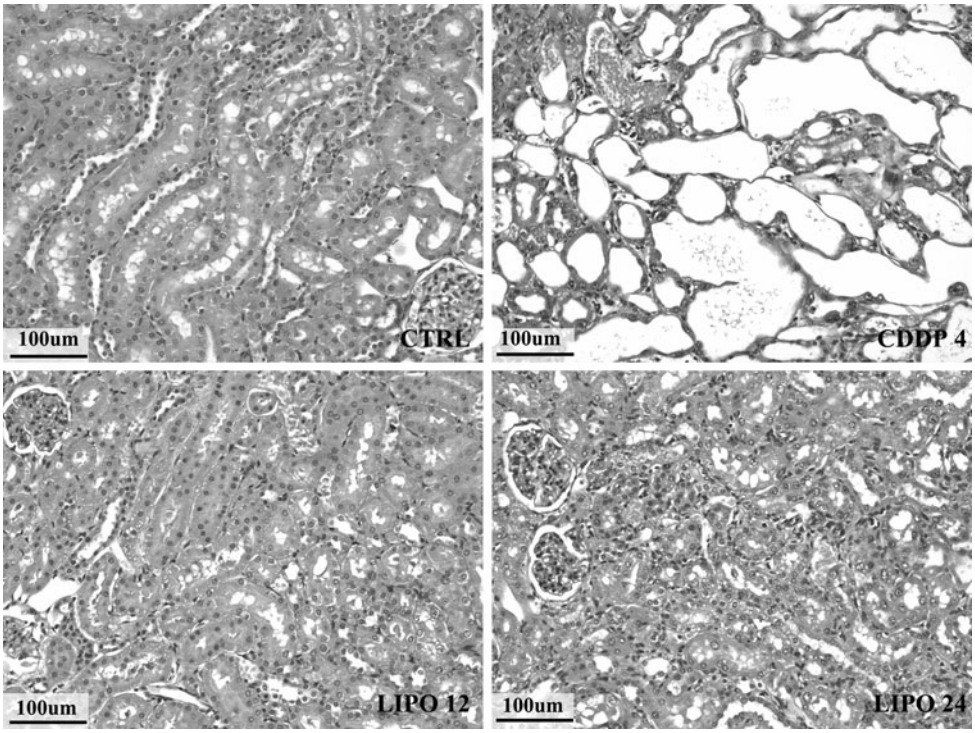

Fig. 1 Histopathology of kidney from CTRL and CDDP 4-, LIPO 12-, and LIPO 24-treated rats, respectively (magnification 10×). CDDP induced from moderate to marked nephropathy with evident tubular toxic changes mainly involving the outer strip of outer medulla in the 100% of the treated rats. Similarly, also LIPO 24 administration was nephrotoxic, but the incidence and the severity of the tubular damage were markedly reduced in comparison with CDDP 4-treated rats. LIPO 12-treated rats did not showed any renal tubular lesions

strip of outer medulla in 100% of the treated rats. These changes were characterized by cystic tubular dilation of proximal tubules with flattened epithelium, multifocal interstitial fibrosis and patterns of tubular regeneration with tubular basophilia, thickened tubular basement membrane, and evident karyomegaly of regenerated tubular epithelial cells (Fig. 1). Similarly, also, LIPO 24 administration was nephrotoxic, but the incidence (5 out of 8) and the severity (from minimal to moderate) of the tubular damage were markedly reduced in comparison with CDDP 4-treated rats (Fig. 1). LIPO 12-treated rats did not showed any renal tubular lesions. Histopathologic examination of the liver specimens showed that both CDDP and Lipoplatin did not induce significant pathological changes on liver parenchyma.

Tissue platinum concentration

The values of tissue platinum concentration in kidney, liver, DRG, and sciatic nerve specimens are reported in Fig. 2. The highest platinum concentration was observed in the kidney and liver for both drugs. The statistical evaluation of platinum concentration in the liver evidenced that there was no significant difference between the two drugs, while platinum concentration in the kidney was significantly higher in LIPO 24-treated rats than in the LIPO 12 and CDDP 4-treated groups ($P < 0.05$ versus CDDP 4 and LIPO 12). The analysis of total platinum accumulation in the sciatic nerve revealed that there was a statistically significant difference in the level of platinum between CDDP

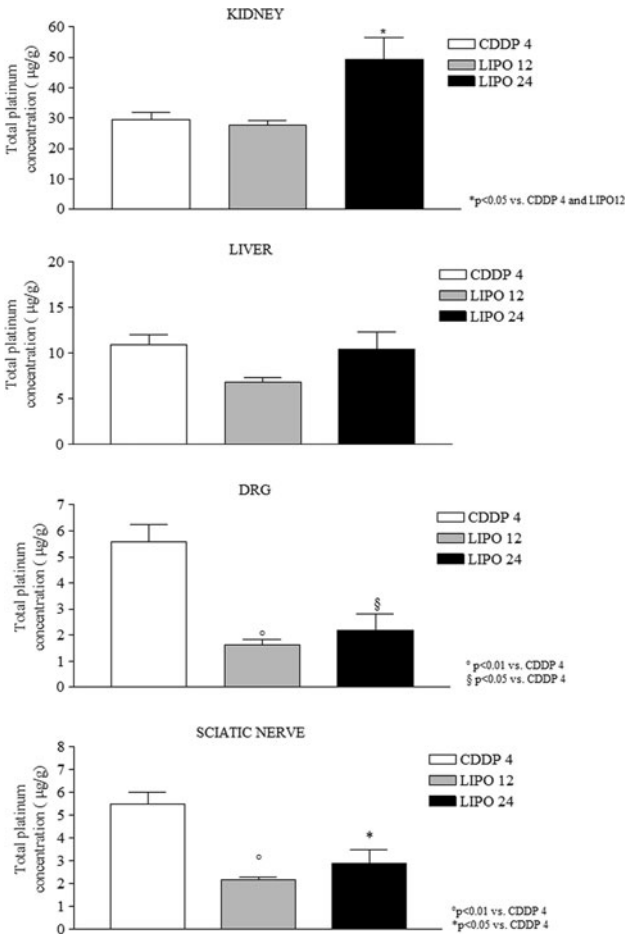

Fig. 2 Total platinum tissue concentration 24 h after the last treatment in kidney, liver, DRG, and sciatic nerve. Platinum concentration is significantly lower in DRG and sciatic nerve after Lipoplatin™ administration

and both doses of Lipoplatin™ ($P < 0.01$ CDDP 4 versus LIPO 12 and $P < 0.05$ CDDP 4 versus LIPO 24), and the same results were observed in the DRG ($P < 0.01$ and $P < 0.05$ versus CDDP 4, respectively).

Neurotoxicity

Neurophysiological evaluation

The neurophysiological evaluation was made after three and four administrations of CDDP and Lipoplatin™. The results obtained during the course of the study are reported in Fig. 3. Statistically significant impairment in NCV ($P < 0.001$) in the CDDP 4- and LIPO 24-treated groups vs. CTRL was observed after the third administration; in these animals, CDDP and LIPO 24 reduced the NCV by about 37 and 34%, respectively. This statistically significant reduction ($P < 0.001$ vs. CTRL) was maintained until the end of the study. By contrast, the treatment with LIPO 12 did not induce a statistically significant impairment in NCV after the third administration, but it became neurotoxic at the end of the treatment period ($P < 0.05$ vs. CTRL), with a reduction in NCV of about 22%.

Histopathology of the sciatic nerve

After treatment, the light microscopic examinations revealed axonopathy with degenerating fibers in the sciatic nerve of rats belonging to all the treated groups. The pathological changes induced by CDDP were overall rather mild, as is usual in this rat model, but they were even milder in the Lipoplatin™-treated animals (Fig. 4).

Histopathology of DRG

As previously described in this well-established model of CDDP-induced peripheral neuropathy [27], at the light microscope, DRG neurons looked smaller in size in the CDDP 4-treated group than in CTRL; multinucleated neurons with eccentric nucleoli were present, but no other evidence of cell damage was observed. The satellite cells had a normal appearance. No evident pathological changes were observed in the neurons or satellite cells in DRG obtained from rats treated with Lipoplatin™, and these results were

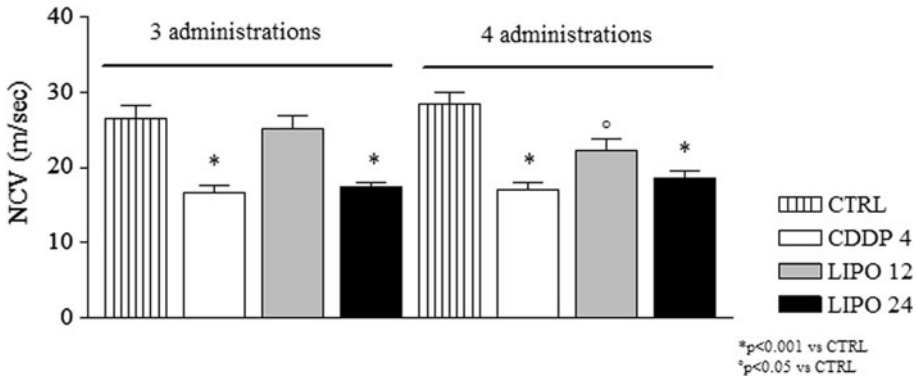

Fig. 3 NCV evaluation after three and four administrations of CDDP and Lipoplatin™. The overall course of NCV changes evidences that LIPO 12 is less neurotoxic than CDDP 4 and LIPO 24

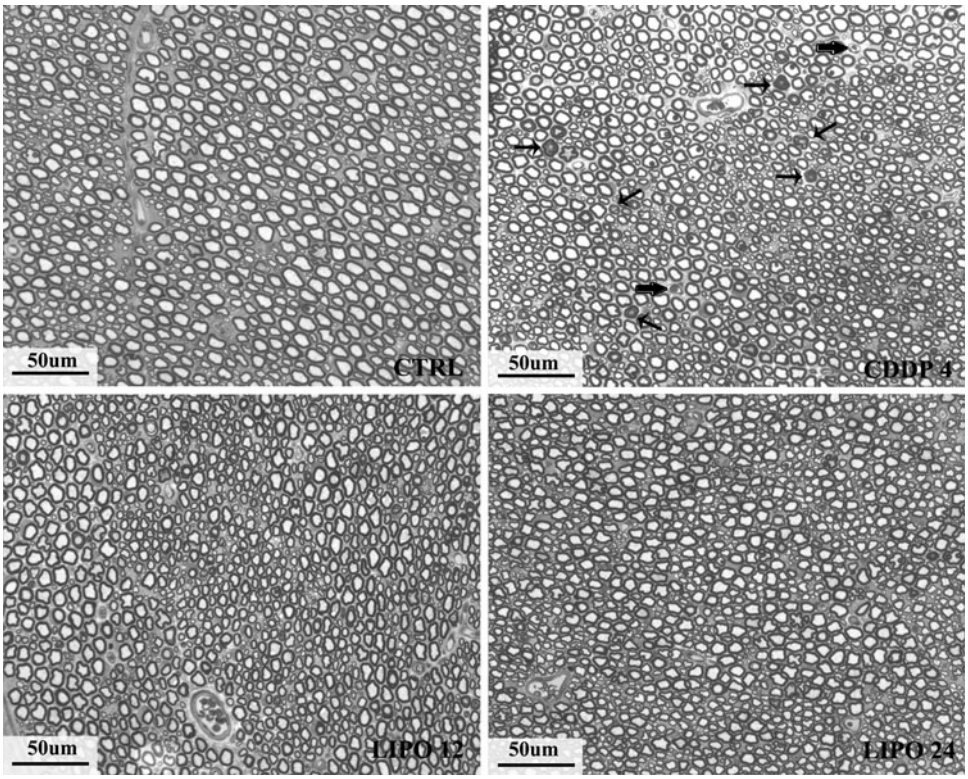

Fig. 4 Histopathology of sciatic nerve from CTRL and CDDP 4-, LIPO 12- and LIPO 24-treated rats, respectively. *Black Arrows* indicate degenerating fibers in randomly selected section (magnification 20×). Also at the pathological level, Lipoplatin™ is clearly less neurotoxic than CDDP 4 at the selected doses

confirmed by the morphometric analysis performed on DRG neurons.

Morphometric analysis of DRG

Based on the documented changes induced by CDDP administration in DRG neurons [26, 27], we performed a morphometric analysis of sensory neurons from CDDP 4-treated animals and we compared the results with those obtained from DRG neurons of Lipoplatin™-treated rats. The results are reported in Fig. 5. The statistical analysis evidenced a significant reduction in somatic, nuclear, and nucleolar areas in DRG of CDDP 4- and LIPO 24-treated rats versus CTRL ($P < 0.001$). However, LIPO 12 induced a less significant atrophy of the soma ($P < 0.001$ vs. CTRL), nucleus and nucleolus in comparison with untreated CTRL rats ($P < 0.05$ vs. CTRL).

Discussion

The efficacy of CDDP in anticancer chemotherapy is dose dependent, but frequently, the use of high doses of the drug to maximize its antitumor activity is not allowed because of nephrotoxicity and peripheral neurotoxicity [15, 16, 28–30]. It is conceivable that newer formulations of platinum compounds with a less toxic effect could play an important role in cancer management [31, 32]. One of these new platinum compounds is the liposomal formulation of CDDP, Lipoplatin™, which consists of soy phosphatidyl choline (SPC-3), cholesterol, dipalmitoyl phosphatidyl glycerol (DPPG), and methoxy-polyethylene glycol-distearoyl phosphatidylethanolamine ($mPEG_{2000}$-DSPE). Lipoplatin™ is composed of 8.9% CDDP and 91.1% lipids (w/w), and its therapeutic efficacy results from its capacity to target primary tumors and metastases, localizing its activity principally in tumor cells [33, 34]. It has been demonstrated that Lipoplatin™ is able to arrest DNA synthesis, to induce oxidative stress and stress signaling as well as apoptotic pathways in tumor cells [12, 35]. Phases I, II, and III clinical studies and animal models have suggested that Lipoplatin™ could substantially reduce the severity of most of the clinically relevant side effects of CDDP while retaining enhanced or similar efficacy [20, 32, 36–38]. However, very limited data are available about the effect of chronic administration of Lipoplatin™ on the peripheral nervous system in comparison with CDDP [21].

For this reason, in the present study, we compared a dose of 4 mg/kg of CDDP, chosen on the basis of the results obtained from our well-established model of peripheral neurotoxicity [27] and on the fact that it is very close to the maximum tolerated dose using the selected schedule and mode of administration, as confirmed by the weight gain curve, with the doses of 12 and 24 mg/kg of Lipoplatin™. The dose of 12 mg/kg was selected on the basis of toxicological data produced in rat models and from activity

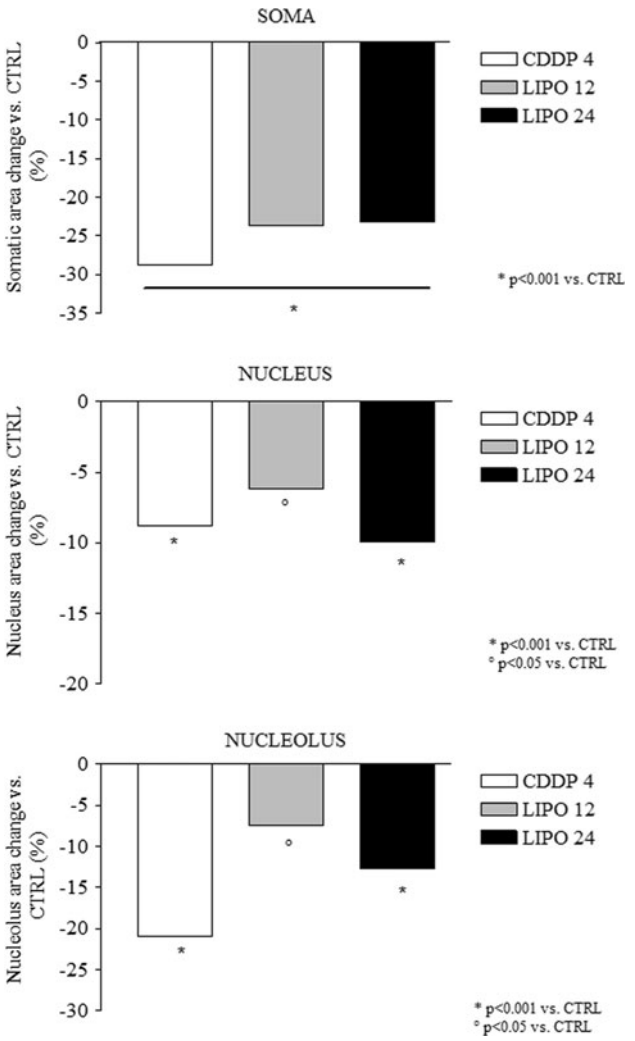

Fig. 5 Morphometric analysis of soma, nucleus, and nucleolus of neurons from DRG. Although the reduction in somatic, nuclear, and nucleolar sizes is evident in all the platinum-treated rats a trend towards a reduced neurotoxic effect of Lipoplatin™ is evident also in DRG neurons

studies conducted in mice xenograft models [20, 37], while the highest dose (24 mg/kg) was used to stress the results of the experiment in terms of possible peripheral neurotoxicity.

Our study demonstrated that Lipoplatin™ at both doses was well tolerated by the animals and confirmed that it is less neurotoxic than CDDP. The apparently conflicting result showing a higher concentration of platinum detected in the kidney after Lipoplatin™ administration despite reduced nephrotoxicity at the histopathological examination is probably simply due to the higher dose and longer half-life of the drug in comparison with CDDP [20, Boulikas T. personal communication]. However, it is important to note that no detrimental effect on kidney function was observed.

At the neurophysiological level, the evaluation of NCV suggested that the highest dose of Lipoplatin™ was equally neurotoxic as CDDP both after 3 and 4 drug administrations, while the lowest dose became neurotoxic only after the fourth administration. Accordingly, the morphometric analysis showed that Lipoplatin™ 12 mg/kg consistently tended to induce less atrophy of the soma, nucleus, and nucleolus sizes of DRG neurons. Both doses of Lipoplatin™ induced a significant reduction in platinum accumulation in the DRG and sciatic nerve versus CDDP. These differences might be explained by a different biodistribution and accumulation of CDDP and Lipoplatin™ in the target organs [20].

In conclusion, the results of our study confirm that effective Lipoplatin™ doses are less nephrotoxic and suggest that they may also be less neurotoxic than CDDP in a dose-dependent manner. Since the clinical implications of these results are potentially extremely important, on this basis further studies in the same animal model aimed at a more precise comparison of the anticancer activity and peripheral neurotoxicity of Lipoplatin™ are warranted.

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