Antitumor activity and biodistribution of cisplatin nanocapsules in nude mice bearing human ovarian carcinoma xenografts

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Cisplatin nanocapsules represent a novel lipid formulation of the anticancer drug cis-diamminedichloridoplatinum(II) (cisplatin), characterized by an unprecedented cisplatin-to-lipid molar ratio, and exhibiting strongly increased in-vitro cytotoxicity compared with the free drug. In this study, antitumor efficacy and biodistribution of PEGylated cisplatin nanocapsules were compared with those of the free drug in a mouse tumor model. Nude mice bearing human ovarian carcinoma OVCAR-3 xenografts were treated twice with a 1-week interval by intravenous administration of cisplatin nanocapsules or cisplatin in solution, and the growth inhibitory effects were determined by measurement of tumor volumes. At a dose of 3 mg cisplatin/kg, corresponding to the maximum tolerated dose of cisplatin nanocapsules, cisplatin nanocapsules and cisplatin in solution exhibited similar therapeutic effectiveness, reducing tumor growth by 90% at day 20 after first injection. The platinum biodistribution was assayed by analyzing plasma and tissues for total platinum content by nonflame atomic absorption spectroscopy. Plasma and tumor concentrations of platinum were similar for both formulations. During the first hour after injection of cisplatin nanocapsules, the platinum content of the kidney was 40% less than that after administering the free drug.

Platinum from nanocapsules showed rapid and 4.5-fold higher accumulation in the liver compared with free cisplatin, and, at a slower rate, accumulation to a high concentration in the spleen. We conclude that the formulation of cisplatin nanocapsules inhibits the growth of OVCAR-3 xenografts in nude mice, albeit to a similar extent as free cisplatin. The results suggest that the antitumor efficacy of the nanocapsules could be improved by preventing rapid clearance from circulation. *Anti-Cancer Drugs* 19:721–727 © 2008 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Introduction

The anticancer drug *cis*-diamminedichloridoplatinum II (cisplatin) is in widespread use for the treatment of a variety of malignancies including genitourinary, head and neck, and non-small cell lung cancers [1]. Cisplatin acts by forming platinum-DNA adducts [2], which interfere with transcription and replication, eventually leading to cell death [3]. The clinical use of cisplatin, however, is restricted by detrimental side effects, including nephrotoxicity, peripheral neuropathy, and ototoxicity [4]. Another disadvantage is the rapid inactivation of cisplatin resulting from complexation with plasma and tissue proteins [5]. Inherent and acquired resistance to cisplatin further limits the application of the drug [6].

To reduce the toxic side effects, to overcome resistance, and to increase the therapeutic index of cisplatin, various nanosize particle formulations of cisplatin have been developed, including lipid-based formulations, the most recent being SPI-077 [7] and lipoplatin [8]. In SPI-077 cisplatin is enclosed in sterically stabilized, PEGylated (stealth) liposomes consisting of hydrogenated soy phosphatidylcholine (PC), cholesterol, and polyethylene-glycol (PEG) derivatized distearoylphosphatidylethanolamine. In preclinical studies, SPI-077 exhibited a prolonged circulation time, increased antitumor efficacy and reduced toxicity compared with free cisplatin [7,9]. In phase I–II clinical trials, however, essentially no antitumor efficacy of SPI-077 was found [10,11]. Lipoplatin did exhibit antitumor efficacy in phase I–II trials with reduced side effects compared with the free drug, and has entered phase III trials [12]. Recently, a liposomal formulation of cisplatin was used to increase the local penetration of cisplatin after inhalation in patients with carcinoma of the lung [13].

A disadvantage of liposomal formulations of cisplatin is the limited bioavailability of the drug in the tumor [14–16]. This is attributed to the low solubility of cisplatin in water, giving rise to liposomal formulations with relatively low drug-to-lipid molar ratios, and the slow kinetics of release. Recently, a method was reported for encapsulating cisplatin in a lipid formulation with an extremely high encapsulation efficiency [17]. The method consists of repeatedly freezing and thawing a concentrated aqueous solution of cisplatin in the presence of equimolar amounts of PC and phosphatidyl-serine (PS), and produces cisplatin nanocapsules, bean-shaped nanoprecipitates of cisplatin coated with a lipid bilayer. The nanocapsules represent a novel lipid formulation of cisplatin characterized by an unprecedented cisplatin-to-lipid molar ratio and by a strongly improved cytotoxicity against tumor cells *in vitro* as compared with the free drug. The formation of the nanocapsules critically depends on the presence of negatively charged phospholipids and positively charged aqua-species of cisplatin [17,18]. Cisplatin nanocapsules have a negative ς potential (– 44 mV) and a hetero-geneous size distribution as determined by dynamic light scattering [17], with 75% of the population having a width around 50 nm and a length between 50 and 250 nm as determined by electron microscopy [19]. Analysis of the contents of the nanocapsules by magic-angle spinning ^{15}N nuclear magnetic resonance revealed that 90% of cisplatin in the solid core of cisplatin nanocapsules is present as the dichlorido species [18].

This study addressed the antitumor efficacy of cisplatin nanocapsules *in vivo*, in nude mice bearing human ovarian carcinoma xenografts. In addition, the biodistribution of cisplatin from nanocapsules was studied and compared with that of the free drug. To improve the stability of the cisplatin PC/PS nanocapsules in mouse serum, nanocap-sules were prepared in which cholesterol and PEG-modified lipids were included in the bilayer coat [20]. Cholesterol is known to reduce membrane permeability, whereas the PEG moieties oriented toward the aqueous solution confer steric stabilization, and should prevent opsonization of nanocapsules and uptake by the reticulo-endothelial system [21]. PEGylation was shown to prolong the lifetime of cisplatin nanocapsules in serum to almost 1 h without adverse effects on cisplatin-to-lipid ratio, size and size distribution, and in-vitro cytotoxicity [20].

Methods

Preparation of cisplatin nanocapsules

Cisplatin (Sigma, St Louis, Missouri, USA) was dissolved in water at a concentration of 5 mmol/l and incubated overnight in the dark at 37°C to ensure full equilibration. Cisplatin nanocapsules were prepared by hydrating dry lipid films (1.2 μmol) consisting of 1,2-dioleoyl-

sn-glycero-3-phosphocholine, 1,2-dioleoyl-sn-glycero-3-phosphoserine, cholesterol (Avanti Polar Lipids Inc., Birmingham, Alabama, USA), and 1,2-distearoyl-sn-gly-cero-3-phosphoethanolamine-*N*-[poly(ethylene glycol)2000] (DSPE-PEG2000; Sigma), in a molar ratio of 27:27: 40:6, with 1.2 ml 5 mmol/l cisplatin in water at 37°C for 30 min. The resulting suspension was subjected to 10 freeze thaw cycles as described [19]. Cisplatin nanocap-sules were collected by centrifugation at low speed (4 min at 2100 rpm) in an Eppendorf centrifuge (Eppen-dorf AG, Hamburg, Germany), washed by resuspension in 1 ml of water and centrifuged as above, and stored at 4°C until use. Platinum contents of cisplatin nanocapsule preparations were quantified by nonflame atomic absorp-tion spectroscopy on a SpectrAA-400 Zeeman spectro-meter (Varian, Palo Alto, California, USA), using a modifier solution of 0.5% Triton X-100 in water, and K_2PtCl_6 as a standard [22]. Batches of cisplatin nanocapsules were characterized for platinum and phos-pholipid contents, yielding the typical platinum/phos-phorus molar ratio of 11 ± 2 [20].

Animals

Female nude mice (Hsd: athymic nude-*nu*) were purchased at the age of 6 weeks (Harlan, Horst, The Netherlands). The animals were maintained in cages with paper filter covers under controlled atmospheric conditions. Cages, covers, bedding, food, and water were changed and sterilized weekly. Animals were handled in a sterile manner in a laminar down-flow hood. Ethics approval was obtained from the ethics committee for animal experiments of the VU University (Amsterdam, The Netherlands) for transplantation of human tumor tissue and for treatment experiments in nude mice. This committee strictly adheres to the standards required by the United Kingdom Coordinating Committee on Cancer Research for the welfare of animals in neoplasia [23].

Antitumor activity

OVCAR-3 ovarian carcinoma xenografts were generated as described [24], and grown subcutaneously in nude mice. For transplantation, tumor fragments with a diameter of 2–3 mm were implanted into both flanks of 8-week-old to 10-week-old nude mice. Upon growth, tumors were measured twice a week in three dimensions with a slide calliper. Tumor volumes (in mm^3) were calculated by the equation *abc*/2, with *a*, *b*, *c* representing tumor length, width, and thickness, respectively. Mice were weighed at the time of tumor measurements.

Treatment was started on day 0 in groups of five mice with a mean tumor volume of approximately 100 mm^3 in each group. Cisplatin in aqueous solution (Bristol-Myers Squibb, Woerden, The Netherlands) and cisplatin nanocapsules were administered intravenously (i.v.) weekly × 2 at doses up to the maximum tolerated dose (MTD). At the MTD, the mice showed a reversible

weight loss up to 15% of the initial weight within 2 weeks after the start of treatment.

In the evaluation of response, tumors that had not reached 20 mm^3 at the start of treatment were considered not evaluable. The increase in tumor volume from the start of treatment (V_0) to the value at any given time (V_t) was calculated for each tumor, and expressed as the relative tumor volume (V_t/V_0) on the day of measurement. The mean of these values was used to calculate the efficacy in percentage as the ratio between treated (T) and control (C) tumors (T/C × 100%).

Biodistribution
Randomized groups of three nude mice bearing OVCAR-3 xenografts with an average tumor volume of 250–300 mm^3 received a single dose of 4 mg/kg of either cisplatin or cisplatin nanocapsules at time zero. At the time-points indicated, the mice were injected with nembutal (150 mg/kg), bled from the axillary vein, and sacrificed by cervical dislocation. Subsequently, liver, kidneys, lungs, spleen, and tumors were collected. Blood was centrifuged and plasma was stored at −20°C until analysis. Tissues were immediately frozen in liquid nitrogen and stored at −80°C until analysis.

For platinum analysis, 100-mg tissue samples were dissolved in 1 ml concentrated nitric acid overnight at 50°C. After addition of 20 μl 0.1 g/ml NaCl, the solvent was evaporated at 100°C under a stream of N$_2$. Residues were dissolved in 1 ml 0.2 mol/l HCl, 0.15 mol/l NaCl by sonication for 10 min, and incubation for 1 h at 50°C. Plasma samples were diluted 1:1 with 0.9% NaCl in 0.4 N HCl. After centrifugation for 2 min at 4000 rpm (Eppendorf centrifuge), 1 ml of 0.1% (v/v) Triton X-100, 0.1% (v/v) antifoam B was added to the supernatants. Total platinum concentration was analyzed by flameless atomic absorption spectrometry using a spectra AA-300 Zeeman AAS (Varian) as described earlier [13,25]. Standards consisting of blank plasma and tissue spiked with platinum were treated in the same way. In these samples, the recovery over the whole concentration range was 104 ± 11%. We tested the recovery in tissues using liver, because it was easily available in a large amount.

The accuracy and precision of the assays for these analyses were 98.4 ± 3.5 and 2.94 ± 3.4%, respectively. Platinum contents were expressed as nmol per gram of wet weight for tissues, and as μmol/l for plasma. The assay was linear over a range of 0.1–10 μmol/l (plasma) and 1.0–100 pmol (tissues). Only calibration curves with a linearity of the slope > 0.990 were considered acceptable. The limit of detection was 0.05 μmol/l (plasma) and 1 pmol (tissues), and the lower limit of quantification was 0.1 μmol/l (plasma) and 1.3 pmol (tissues). Areas under the curve were calculated from the plots of mean concentration versus time using the trapezoidal rule.

Statistics
Antitumor activity and differences in the exposure of tissues to platinum were evaluated by Student's *t*-test.

Results
Antitumor activity of cisplatin nanocapsules
The in-vivo antitumor efficacy of cisplatin nanocapsules was tested in mice bearing OVCAR-3 xenografts, of which the sensitivity to cisplatin is well established [24]. Cisplatin in solution and the cisplatin nanocapsules were injected twice with a 1-week interval up to 5 mg cisplatin/kg, corresponding to the MTD for cisplatin [26]. The weight loss caused by cisplatin nanocapsules at 3 mg/kg was slightly, but not significantly, stronger than that induced by the corresponding dose of free drug ($P = 0.1$) (Table 1). Fourteen days after first treatment with the doses tested, the loss of weight was fully reversed. Higher doses of cisplatin nanocapsules up to 5 mg/kg led to a reversible weight loss up to 25% within 2 weeks of treatment (data not shown), and were therefore considered too toxic. As a consequence, the antitumor activity was compared up to a dose of 3 mg/kg for both formulations.

Treatment with 1.5 mg/kg cisplatin delayed xenograft growth by 18 days (Table 1). A dose of 3 mg/kg cisplatin induced regression of the tumors to 30% of the initial tumor volume after 20 days, after which the tumors resumed growth. Cisplatin administered in nanocapsules delayed tumor growth and induced regression, albeit slightly less effectively than the corresponding doses of

Table 1 Activity of cisplatin nanocapsules compared with cisplatin in nude mice bearing OVCAR-3 xenografts

Treatment weekly x2 (mg cisplatin/kg)	Maximum weight loss ± SD (%) (day)	Weight day 14 ± SD (%)	Toxic deaths	Optimum T/C (%)[a,b] (day) (tumors)
Cisplatin				
1.5	0	105.4 ± 2.4	0/5	26** (18) (7)
3	4 ± 5.5 (10)	103.4 ± 1.9	0/5	8*** (20) (8)
Nanocapsules				
1.5	3.4 ± 13.5 (7)	103.7 ± 9.1	0/5	65* (5) (8)
3	9.0 ± 2.5 (10)	99.5 ± 3.2	0/5	12*** (20) (9)

[a]T/C (%), optimal value of the mean of relative tumor volume in treated group/mean of relative tumor volume in control group × 100, calculated within 5 weeks after completing chemotherapy.
[b]Significant differences, *$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$, between treated and control animals evaluated by Student's *t*-test.

the free drug. At 1.5 mg/kg cisplatin nanocapsules, tumor growth was delayed by 14 days, whereas at 3 mg/kg the tumor volume was reduced to 41% of the initial volume on day 20 after the first injection. The difference in antitumor activity between cisplatin nanocapsules and the free drug at 3 mg/kg was not significant ($P = 0.23$; Table 1).

Biodistribution of platinum from cisplatin nanocapsules
To gain insight into the fate of the cisplatin nanocapsules after i.v. administration, the platinum concentrations in plasma and several tissues were determined at different time-points up to 24 h after a single i.v. injection of cisplatin and cisplatin nanocapsules at a dose of 4 mg cisplatin/kg (Fig. 1). As it concerned a single dose, it was

chosen above the MTD of cisplatin nanocapsules to facilitate detection. The free drug was rapidly cleared from circulation by the kidney, the excretory organ of cisplatin that showed higher platinum contents than tumor, liver, lungs, and spleen at all time-points, in agreement with previous studies [7,15,16]. Administration of cisplatin nanocapsules did neither prolong the circulation time of cisplatin, nor did it enhance the accumulation of platinum in the tumor, compared with administration of cisplatin in solution (Fig. 1, panels a and b). The data suggest that during the first hour after injection, removal of platinum from nanocapsules from the blood stream by the kidneys was slightly less than that of the free drug (Fig. 1c). In contrast, already 20 min after injection, cisplatin from nanocapsules showed strong

Fig. 1
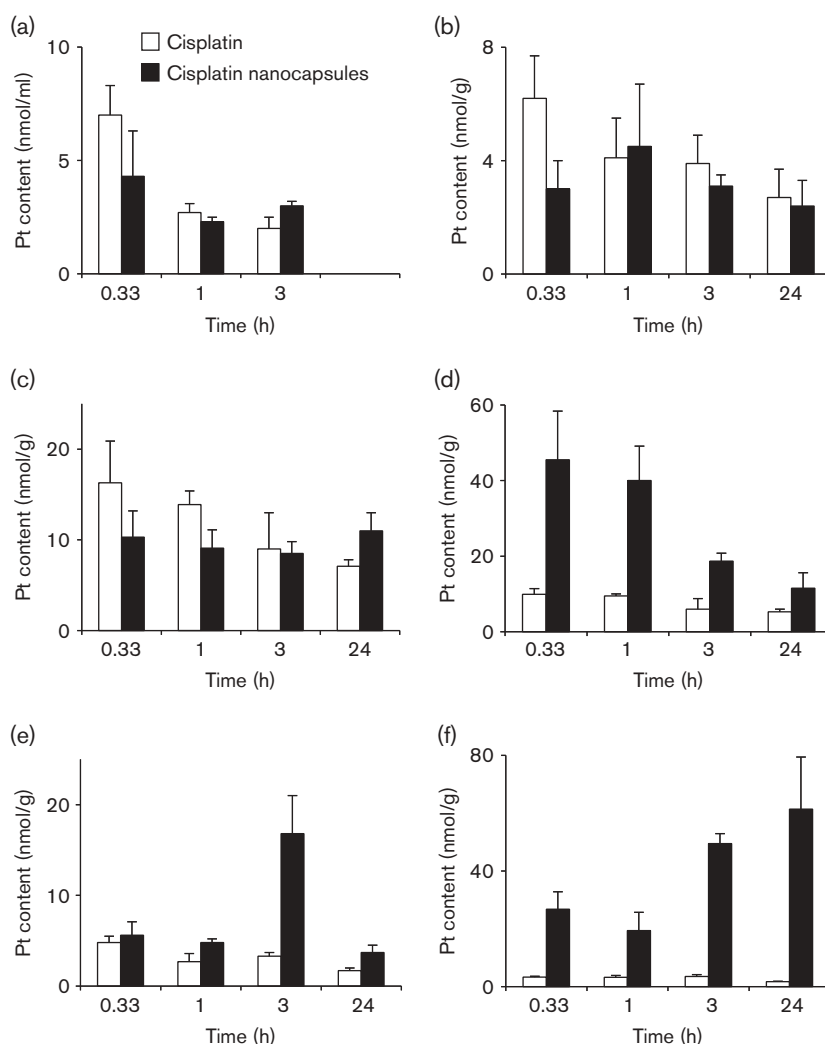
Pharmacokinetic profile of platinum (Pt) in plasma (a), tumor (b), kidney (c), liver (d), lung (e), and spleen (f) of nude mice bearing OVCAR-3 tumors, following a single intravenous dose of cisplatin or cisplatin nanocapsules corresponding to 4 mg cisplatin/kg. The Pt content in plasma at 24 h was not determined. Values are expressed as mean ± SD ($n = 3$).

Table 2   Exposure of different tissues to platinum after administration of cisplatin at 4 mg/kg in solution or in nanocapsules to nude mice bearing OVCAR-3 xenografts

Tissue	Cisplatin[a]	Cisplatin nanocapsules[a]
Plasma[b]	0.5 ± 0.1	0.5 ± 0.1
Tumor	5.0 ± 0.9	4.0 ± 0.5
Kidney	12.1 ± 3.0	13.8 ± 2.3[c]
Liver	8.4 ± 2.0	24.0 ± 4.0[c]
Lung	3.6 ± 0.5	12.4 ± 1.1[c]
Spleen	3.7 ± 0.6	75.2 ± 10.4[c]

[a]Area under the curve from 20 min up to 24 h, expressed as µmol.min/g or ml (mean ± SD, $n = 3$).
[b]Determined up to $t = 3$ h.
[c]Higher exposure to platinum ($P < 0.01$) from nanocapsules than from solution.

accumulation in the liver, with a platinum level 4.5-fold higher than that in cisplatin-treated mice (Fig. 1d). At later time-points peak levels of platinum from cisplatin nanocapsules were measured in the lung at 3 h (Fig. 1e), and in the spleen (Fig. 1f) in which the platinum content increased up to 24 h after injection. The exposure of the liver, lung, and spleen to cisplatin as measured by the areas under the curve was increased 3-fold, 3.5-fold, and 20-fold, respectively, with the nanocapsule formulation as compared with treatment with cisplatin in solution (Table 2).

Discussion

Cisplatin nanocapsules present a new lipid formulation of the anticancer drug cisplatin, in which an essentially water-free nanoprecipitate of the drug is covered by a single lipid bilayer [17,18]. This study reports the first in-vivo results obtained with cisplatin nanocapsules. The therapeutic effectiveness and the biodistribution of cisplatin nanocapsules were compared with those of cisplatin in solution in nude mice bearing human ovarian carcinoma OVCAR-3 xenografts.

At the doses tested, cisplatin nanocapsules and the free drug exhibited similar antitumor efficacies, consistent with the similar levels of accumulation of platinum in the tumor obtained with the two formulations. As the lifetime of cisplatin nanocapsules in mouse serum *in vitro* is limited to approximately 1 h [20], it could be argued that the similarity in antitumor efficacy and accumulation in the tumor of the two formulations is owing to an immediate and complete release of content of the nanocapsules upon i.v. administration. However, the different biodistribution patterns of platinum from the two formulations indicate otherwise. Whereas the kidneys cleared the free drug from circulation, platinum from cisplatin nanocapsules rapidly accumulated in the liver, and more gradually in the lung and the spleen, indicating that at least part of the nanocapsules remained in circulation beyond 3 h after injection. The results imply that part of the inhibition of tumor growth observed upon administering cisplatin nanocapsules must originate from nanocapsules accumulating in the tumor, and, as a consequence, that once in the tumor, the nanocapsules release cisplatin to exert their cytotoxic effect. We conclude that cisplatin nanocapsules are effective in inhibiting tumor growth.

The antitumor efficacy of the cisplatin nanocapsules *in vivo* reported here does not correlate with the in-vitro data, which demonstrated a strong increase in cytotoxicity of cisplatin nanocapsules compared with the free drug [17]. Possible explanations include differences in stability of the nanocapsules in tissue culture medium and in plasma, differences in cell accessibility between cells cultured *in vitro* and cells in a tumor, and differences in the mechanism of cellular drug uptake. Whereas the uptake of free cisplatin released from the nanocapsules in the interstitial space is likely to predominate in the tumor [27], endocytosis of intact nanocapsules accounts for the increased cytotoxicity *in vitro* (our unpublished results).

The biodistribution of cisplatin nanocapsules appeared to be different from that of SPI-077, a PEGylated liposomal formulation of cisplatin, in tumor-bearing mice [7,15,16]. SPI-077 liposomes exhibit a prolonged circulation time compared with the free drug, and increased accumulation in the tumor. Furthermore, the relative rate of accumulation of platinum from SPI-077 in the liver and spleen is slower and the extent of accumulation relative to that of the free drug is less compared with platinum from cisplatin nanocapsules. A number of factors are likely to account for the different biodistribution patterns of platinum from nanocapsules and from SPI-077. First, contrary to cisplatin nanocapsules, SPI-077 liposomes hardly release contents in plasma [7,16,19]. Next, unlike SPI-077, cisplatin nanocapsules have a negative surface charge originating from the anionic phospholipid PS that cannot be completely shielded by PEGylation [20], and is known to enhance removal from circulation by the reticulo-endothelial system [21], and by the liver in particular [28]. Particle size is another important parameter determining the rate of clearance from circulation. Whereas SPI-077 liposomes have an average particle size of 110 nm and a narrow size distribution [7], preparations of nanocapsules are more heterogeneous and contain larger particles [17,19,20] that are more prone to clearance [29]. Furthermore, the different physical properties of the particles, that is, the solid core of the nanocapsules versus the aqueous interior of the liposomes, may influence pharmacokinetics. Finally, the rate of clearance depends on the amount of lipid given. The amount of lipid administered with SPI-077 is at least two orders of magnitude higher than the amount of lipid contained in a corresponding dose of cisplatin nanocapsules owing to the huge difference in drug-to-lipid ratios between the formulations (*cf.* [7,19]). As a consequence, the doses of SPI-077 generally used are expected to saturate the mechanism(s) of clearance, in contrast to what can be expected for the corresponding doses of

cisplatin nanocapsules [21,30,31], providing an additional explanation for the different rates of clearance.

The differences in pharmacological behavior between SPI-077 and our formulation suggest that the accumulation of cisplatin in the tumor and the antitumor efficacy could be improved by increasing nanocapsule stability in plasma, and by preventing the rapid scavenging by the liver and spleen. The latter may be accomplished by reducing the negative surface charge and/or the particle size of the cisplatin nanocapsules, or by saturating the mechanism(s) of clearing by administering liposomes before the nanocapsules.

The importance of the particles' surface charge in determining the biodistribution is further suggested by comparison of our results with those recently reported for cisplatin-loaded hydrophobically modified glycol chitosan nanoparticles [32]. These particles carry a positive surface charge, and with a mean diameter of 300–500 nm, they are larger than our cisplatin nanocapsules. Upon i.v. administration in tumor-bearing mice, the cisplatin-loaded hydrophobically modified glycol chitosan nanoparticles did not end up in the liver and the spleen, but accumulated in the tumor, exhibiting reduced toxicity and improved antitumor efficacy compared with free cisplatin [32].

Alternatively, cisplatin nanocapsules could be useful for applications such as sustained drug release after intraperitoneal administration for treatment of peritoneal deposits, or infusion into the hepatic artery in case of liver metastases. Previously, favorable anticancer activities were reported for intraperitoneally administered, cisplatin-releasing, polymer-based microspheres [33] and nanoparticles [34].

Interestingly, the application of the nanocapsule technology to the cisplatin-derived anticancer drug carboplatin recently yielded a formulation with a drug-to-lipid ratio that is 15-fold lower than that of cisplatin nanocapsules [35]. Carboplatin nanocapsules can be sized by extrusion and prepared with lower PS contents, rendering such a formulation promising for in-vivo application.

Acknowledgements

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