Carboplatin nanocapsules: a highly cytotoxic, phospholipid-based formulation of carboplatin

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Abstract

Platinum-based drugs are widely used in cancer chemotherapy. However, their clinical use is limited by systemic toxicity, rapid blood clearance, and the occurrence of resistance. Our research is aimed at increasing the therapeutic index of these drugs by encapsulation in a lipid formulation. Previously, we developed a method for efficient encapsulation of cisplatin in a lipid formulation, yielding cisplatin nanocapsules. Here, we show that carboplatin, a cisplatin-derived anticancer drug with different chemical properties, can be efficiently encapsulated in a lipid formulation by a similar method. The carboplatin nanocapsules exhibit a very high cytotoxicity *in vitro*: the IC_{50} value of carboplatin nanocapsules is up to a 1,000-fold lower than that of conventional carboplatin when tested on a panel of carcinoma cell lines. Cellular platinum content analysis and confocal fluorescent imaging of the interaction of the carboplatin nanocapsules with IGROV-1 cells indicate that the improved cytotoxicity is due to increased platinum accumulation in the cells, resulting from uptake of the formulation by endocytosis. [Mol Cancer Ther 2006;5(8):2007–12]

Introduction

Platinum-based drugs are widely used as anticancer agents in the clinic, particularly against ovarian and lung cancer (1). The most commonly used platinum-based drug, *cis*-diamminedichloroplatinum(II) (cisplatin), is activated via hydrolysis, yielding positively charged, very reactive aqua species that can form DNA adducts and thus cause cell death (1). Common problems associated with the clinical use of cisplatin are cumulative toxicities of nephrotoxicity, ototoxicity, and peripheral neuropathy (2, 3). In addition to the serious systemic toxicities, rapid blood clearance and inherent or treatment-induced resistant tumor cell subpopulations limit the therapeutic efficacy of cisplatin (1).

The adverse effects of cisplatin prompted a parallel synthesis effort to design more effective and less toxic analogues. It was found that modification of cisplatin to contain less labile leaving groups alters the pharmacokinetics and the toxicity profile of the drug. Replacement of the chloride leaving groups with a cyclobutane-dicarboxylato ligand, forming *cis*-diammine-cyclobutane-1,1-dicarboxylatoplatinum(II) (carboplatin), slowed down the rate of hydrolysis 100-fold, rendering the compound less reactive and reducing systemic toxicities, whereas antitumor activity was retained (1, 4, 5). At effective doses, carboplatin produced substantially less nausea, vomiting, nephrotoxicity, and neurotoxicity than cisplatin, and bone marrow suppression was its predominant toxicity. However, the problems of intrinsic and acquired resistance persist, thereby limiting the clinical use of carboplatin (1–3). One strategy that can be used to overcome the side effects and occurrence of resistance to platinum drugs is to encapsulate them in polymer formulations or in liposomes.

Liposomes are vesicles, composed of one or more phospholipid bilayers surrounding an aqueous lumen (6). Systemic treatment with liposomes leads to extravasation and accumulation of liposomal drugs within neoplastic tissues because of the leaky vasculature and scarce lymphatic vessels in tumors (7, 8). Liposomally targeted cytotoxic drugs are becoming an established tool in the treatment of cancer. The liposomal anthracycline agents (doxorubicin and daunorubicin) have shown that these formulations can alter the efficacy and toxicity profiles of the parent compound (9–12). Following these initial successes, liposomal formulations of other agents (cisplatin and vincristine) have entered (pre)clinical trials (13–17), and lipid-encapsulated camptothecins (18, 19) and topoisomerase inhibitors (20) are under investigation.

Previously, we developed a novel method for the efficient encapsulation of cisplatin in a lipid formulation (21). This method generates a novel type of structure, bean-shaped cisplatin nanoprecipitates surrounded by a single phospholipid bilayer. Cisplatin nanocapsules have an unprecedented cisplatin-to-lipid ratio and an *in vitro* cytotoxicity up to 100-fold higher than the free drug (21, 22). The method of encapsulation takes advantage of the limited solubility of cisplatin in water (~8 mmol/L at 37°C;

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ref. 23). In the model proposed for the formation of cisplatin nanocapsules, freezing induces the formation of small positively charged cisplatin aggregates, which interact with and are somehow covered by the negatively charged lipid bilayers (21, 22).

Carboplatin is chemically distinct from cisplatin, in that it is more water soluble (~40 mmol/L at 37°C; ref. 24), and its rate of aquation is much slower (4, 5). Here, we report that, despite the differences in chemical properties, carboplatin can be efficiently encapsulated in a lipid formulation by a similar method, resulting in a formulation with a strongly improved cytotoxicity against tumor cells *in vitro* compared with the free drug.

Materials and Methods

Preparation and Characterization of Carboplatin Nanocapsules

Carboplatin (ABCR Gmbh & Co. KG, Karlsruhe, Germany) was dissolved at 5 mmol/L in Milli-Q water and incubated overnight in the dark at 37°C. Lipid dispersions (1 mmol/L) were prepared by adding the carboplatin solution to a dry film of phospholipids (Avanti Polar Lipids, Birmingham, AL) with a composition as indicated. The lipid dispersions were then incubated at 37°C for 30 minutes followed by 10 freeze-thaw cycles using ethanol/dry ice (−70°C) and a water bath (55°C). Extravesicular carboplatin was removed by centrifugation (twice, 4 minutes, 20°C, and 20,800 × *g*), resuspending the pellet in Milli-Q. Where indicated, pellet fractions were extruded (Anotop 10, 0.2-μm pore size; Whatman, Brentford, Middlesex, United Kingdom), and reisolated at higher centrifugation speed (70,000 × *g*). Platinum and phospholipid content was quantified as described (21, 25, 26). Particle size was determined on a Zetasizer 3000 (Malvern Instruments, Malvern, United Kingdom).

Cell Culture

The IGROV-1, OVCAR-3, A2780, and A498 cell lines were maintained in RPMI 1640 (Life Technologies, Carlsbad, CA) supplemented with 10% FCS Gold (PAA Laboratories GmbH, Pasching, Austria) and 100 units/mL penicillin and 100 μg/mL streptomycin. NCI-H522 was cultured in the same medium with extra glucose (final concentration of 4.5 g/L) and 1 mmol/L sodium pyruvate and SK-OV-3 cells in DMEM (Life Technologies) supplemented with 10% FCS Gold, 100 units/mL penicillin, and 100 μg/mL streptomycin.

In vitro Cytotoxicity Assay

Cytotoxicity was assayed as described (21). In short, approximately 500 to 1,000 human carcinoma cells were seeded per well in a 96-well plate (Costar, Corning, NY) in 200 μL growth medium. After 24 hours, carboplatin formulations were added at the concentrations indicated, and the cells were incubated for 4 days at 37°C. Tumor cell survival was measured in a sulforhodamine B assay (27). The data were fitted to a sigmoidal dose-response curve (variable slope) using GraphPad Prism (GraphPad Software, San Diego, CA).

Intracellular Platinum Accumulation

The intracellular platinum accumulation was studied using ~10^7 cells. The cells were incubated with conventional carboplatin or carboplatin nanocapsules for 2 hours at concentrations of 1, 10, and 50 μmol/L. Immediately afterwards, the cells were washed twice with ice-cold PBS, harvested by scraping in ice-cold PBS, centrifuged, and resuspended in 1.0 mL Milli-Q. A sample was taken for protein determination (Bradford), and 800 μL were dried by overnight centrifugation under vacuum. The cell pellet was digested in 65% (v/v) nitric acid at 75°C for 2 hours. After dilution in water, platinum content was analyzed by nonflame atomic absorption spectroscopy. Cellular platinum levels were expressed in nanogram platinum per milligram protein.

Confocal Fluorescence Microscopy

IGROV-1 cells were seeded on glass coverslips in culture medium. For preparation of fluorescent nanocapsules, two changes in the standard protocol for preparation of nanocapsules were made: carboxyfluorescein (0.5 mmol/L) was added to the carboplatin solution and Bodipy-C5-phosphatidylcholine (0.2 mol%; Molecular Probes, Carlsbad, CA) was incorporated in the lipid film. The cells were incubated for 1 hour with double-labeled nanocapsules (final concentration of carboplatin, 50 μmol/L) and fixed with 4% paraformaldehyde for 15 minutes at room temperature. The coverslips were mounted in Mowiol/Dabco [Calbiochem (San Diego, CA)/Sigma (St. Louis, MO)] and images were collected by confocal microscopy (Nikon C1 microscope; Nikon, Tokyo, Japan).

Results and Discussion

Membrane Encapsulation of Carboplatin Strongly Improves Its Cytotoxicity Toward a Panel of Human Cancer Cell Lines

The method to encapsulate carboplatin involves hydration of a dry lipid film composed of equimolar amounts of zwitterionic 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and anionic 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (DOPS) with 5 mmol/L carboplatin in Milli-Q water followed by 10 freeze-thaw cycles and removal of unencapsulated drug by centrifugation. The resulting lipid formulation of carboplatin was extremely cytotoxic toward IGROV-1 ovarian carcinoma cells (Fig. 1; Table 1) with a typical IC_{50} (drug concentration at which 50% of the cells survive and grow) of ~1.7 nmol/L. The IC_{50} value for the free drug was 2.0 μmol/L. A lipid suspension not loaded with carboplatin (blank) was not cytotoxic, and mixing conventional carboplatin with the blank lipid suspension did not increase the cytotoxicity of carboplatin (data not shown). In addition, toward the SK-OV-3 and A2780 ovarian carcinoma cell lines, which are frequently used to test new platinum-based drugs *in vitro* and *in vivo* (28–30), the lipid formulation of carboplatin exhibited strongly improved cytotoxicity compared with the free drug (Fig. 1; Table 1). Moreover, the NCI-H522 non–small lung carcinoma cell line, which is not very sensitive to conventional

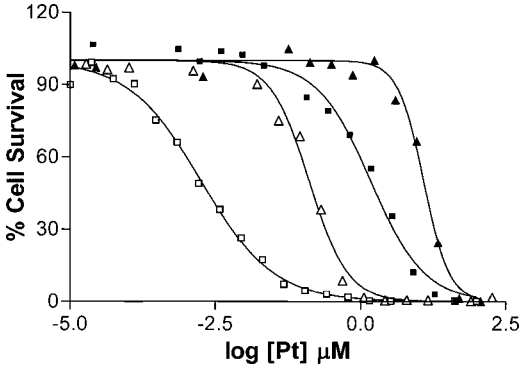

Figure 1. Cytotoxicity of carboplatin lipid formulation toward human ovarian carcinoma cell lines. The toxicity of the DOPC/DOPS (1:1) lipid formulation of carboplatin (□ and Δ) and conventional carboplatin (■ and ▲) was measured *in vitro* toward IGROV-1 (■ and □) and SK-OV-3 (▲ and Δ) cells. Representative experiment.

carboplatin (31), and the A498 renal carcinoma cell line are effectively killed by addition of the carboplatin lipid formulation (Table 1). These results show that the lipid formulation of carboplatin is much more effective than conventional carboplatin in killing the cell lines tested. In addition, they show that some cell lines, which are relatively insensitive for carboplatin, can be killed with comparatively low concentrations of lipid-encapsulated carboplatin, indicating that the lipid formulation not only improves the therapeutic effect but also may increase the therapeutic profile of carboplatin.

Mechanism of Toxicity

To explain the increased cytotoxicity of the lipid formulation, cellular platinum accumulation was measured by nonflame atomic absorption spectroscopy. For both conventional and encapsulated carboplatin, an apparently nonsaturable relationship between the concentration of carboplatin administered and the intracellular platinum concentrations (expressed in nanogram platinum/milligram protein) in IGROV-1 ovarian carcinoma cells was observed after 2 hours of treatment (Fig. 2A). In the range of 1 to 50 μmol/L, the platinum accumulation in cells treated with lipid-encapsulated carboplatin was much higher than in cells treated with the free drug, with the platinum accumulation in cells treated with 1 μmol/L lipid-formulated carboplatin being comparable with the platinum accumulation in cells treated with 50 μmol/L conventional carboplatin. At lower concentrations, in the range of the effective dose of carboplatin nanocapsules, we were unable to measure the cellular carboplatin accumulation in cells treated with free carboplatin because the platinum concentation in the samples was below the detection limit of the nonflame atomic absorption spectroscopy. However, it was possible to measure the platinum accumulation in cells treated with encapsulated carboplatin at a concentration of 20 nmol/L, which is only a factor of 10 above the IC_{50} value (Table 1). Carboplatin (20 nmol/L) administered as nanocapsules yielded a similar cellular platinum accumulation as 1 μmol/L free carboplatin (data not shown), consistent with the results obtained in the higher concentration range, suggesting that the mechanism of uptake is independent of the concentration. The results indicate that the increased efficacy of carboplatin nanocapsules compared with the free drug can be explained by an increased cellular accumulation of the encapsulated drug.

Next, we investigated whether the differences in efficacy of the carboplatin nanocapsules between the cell lines tested (Table 1) were due to differences in cellular accumulation. IGROV-1 (IC_{50} value of nanocapsules is 1,000 times lower than the IC_{50} value of the free drug), SK-OV-3 (IC_{50} value of nanocapsules is 120 times lower), and A2780 cells (IC_{50} value of nanocapsules is 10 times lower) were treated with 30 μmol/L carboplatin, free or encapsulated, and the cellular platinum accumulation was measured. Interestingly, the differences found in the cellular platinum accumulation did not correspond with the differences in the ratios of the IC_{50} values (Fig. 2B). The cellular platinum accumulation in cells treated with free carboplatin was similar in the three cell lines, and the increase in platinum accumulation on treatment with carboplatin nanocapsules was similar in the SK-OV-3 and A2780 cells and only slightly lower than in IGROV-1 cells (Fig. 2B). These results show that the platinum accumulation does not explain the differences in cytotoxicity

Table 1. IC_{50} values of the carboplatin lipid formulation and conventional carboplatin toward human carcinoma cell lines

Cell line	Origin	IC_{50}, $n \geq 3$ (μmol/L ± SE)		Fold difference in IC_{50}*
		Conventional	Encapsulated	
IGROV-1	Ovarian carcinoma	2.0 ± 0.4	0.0020 ± 0.0004	1,000
SK-OV-3	Ovarian carcinoma	11.7 ± 0.4	0.10 ± 0.02	120
A2780	Ovarian carcinoma	4.4 ± 0.3	0.44 ± 0.03	10
A498	Renal cell carcinoma	7.4 ± 0.8	0.037 ± 0.004	200
NCI-H522	NSC lung carcinoma	24.9 ± 3.0	0.12 ± 0.01	200

NOTE: The cell survival in response to increasing concentrations of carboplatin was measured in a sulforhodamine B assay (see Materials and Methods) for conventional and encapsulated carboplatin, and IC_{50} values were determined for the cell lines listed.
*The ratio of the average IC_{50} value of conventional over that of lipid-encapsulated carboplatin.

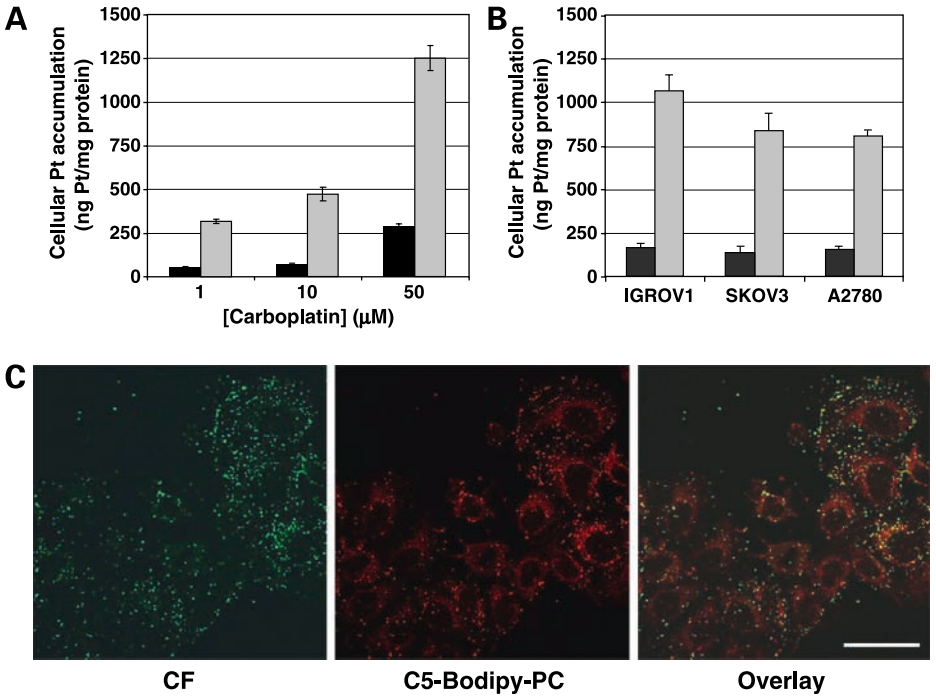

Figure 2. Mechanism of cytotoxicity. **A,** the accumulation of platinum in IGROV-1 cells treated with encapsulated carboplatin (*gray columns*) is much higher than in cells treated with corresponding concentrations of conventional carboplatin (*black columns*). *Columns,* cellular Pt accumulation (n = 3); *bars,* SD. **B,** the accumulation of platinum in IGROV-1, SK-OV-3, and A2780 cells treated with 30 μmol/L encapsulated carboplatin (*gray columns*) is much higher than in cells treated with a corresponding concentration of conventional carboplatin (*black columns*). *Columns,* cellular Pt accumulation (n = 3); *bars,* SD. **C,** uptake of the lipid formulation of carboplatin in IGROV-1 cells studied by confocal fluorescence microscopy. Cells were incubated for 60 min with double-labeled carboplatin particles [Bodipy-C5-phosphatidylcholine (*C5-Bodipy-PC*) in bilayers/carboxyfluorescein enclosed] to a final carboplatin concentration of 50 μmol/L. Bar, 50 μm.

between the cell lines of encapsulated versus free drug and indicate that differences in processing of the carboplatin from nanocapsules determine the efficacy of carboplatin nanocapsules in different cell lines.

To study the uptake of the encapsulated carboplatin, confocal fluorescence microscopy studies were done (Fig. 2C), in which the carboplatin-containing particles were labeled by encapsulating carboxyfluorescein in the lumen and inserting Bodipy-C5-phosphatidylcholine in the lipid coats. The Bodipy-C5-phosphatidylcholine and carboxyfluorescein labels partly colocalize within the cell, suggesting uptake via endocytosis, consistent with the generally accepted view that the cellular uptake of liposomes is mediated by adsorption of liposomes onto the cell surface and subsequent endocytosis (32). Together, these data show that the increased intracellular accumulation of carboplatin through endocytosis is likely to account for the increased cytotoxicity of the encapsulated carboplatin.

Characterization of the Carboplatin Lipid Formulation

To characterize the lipid formulation of carboplatin, we determined the average carboplatin and phospholipid yields, the drug-to-lipid molar ratio (Pt/Pi) and the particle size of the formulation. Using the standard protocol, 2.6 ± 0.1% of the carboplatin and 15.8 ± 0.7% of the phospholipid were recovered in the pellet, resulting in a formulation with a drug-to-lipid molar ratio of 0.71 ± 0.03 (n = 20; Table 2) and an average size of 290 nm (polydispersity index, 0.08). Assuming a trapped volume of 5 L/mol of phospholipid based on the size of the particles (33), a drug-to-lipid molar ratio of 0.71 translates to an intravesicular carboplatin concentration in excess of 140 mmol/L. This value exceeds the solubility limit of carboplatin, suggesting that at least

Table 2. Factors determining carboplatin nanocapsule formation and cytotoxicity on IGROV-1 cells

Lipid composition	Changes in the standard protocol*	Pt/Pi ratio, $n \geq 4$ (±SD)	Cytotoxicity†
DOPC/DOPS (1:1)	—	0.71 ± 0.03	+++
DOPC/DOPS (1:1)	Without freeze-thaw	0.06 ± 0.07	–
DOPC/DOPS (1:1)	Preformed liposomes	0.54 ± 0.05	+++
DOPC/DOPS (1:0)	—	0.12 ± 0.05	–
DOPC/DOPG (1:1)	—	0.73 ± 0.23	–
DOPC/DOPS (1:4)	—	0.71 ± 0.09	++
DOPC/DOPS (4:1)	—	0.54 ± 0.04	++

NOTE: The molar ratio of platinum over phospholipid phosphorous (Pt/Pi) and the cytotoxicity of the pellet fractions were measured.
*Hydrate dry lipid film with 5 mmol/L carboplatin in H_2O and freeze-thaw.
†+++, >1000-fold increase; ++, >100-fold increase; and –, no significant increase in cytotoxicity compared with conventional carboplatin.

part of the carboplatin in the formulation is present as a solid substance.

Omitting the freeze-thaw cycles resulted in a dramatic decrease in cytotoxicity (Table 2). The decrease in cytotoxicity was paralleled by a decrease in encapsulation efficiency (Table 2), suggesting a direct relation between drug-to-lipid ratio and cytotoxicity. In addition, we found that the stage at which carboplatin is added was not critical: carboplatin addition to a dry lipid film or to preformed liposomes both yielded pellet fractions with similar carboplatin encapsulation efficiencies and cytotoxicities (Table 2).

Hydration of a phosphatidylcholine lipid film with carboplatin followed by 10 freeze-thaw cycles resulted in a formulation, in which carboplatin was not encapsulated efficiently and which was not significantly more cytotoxic than the free drug (Table 2). This indicates a requirement for anionic lipids, which is most likely based on an electrostatic interaction of the anionic phosphatidylserine with a positively charged species of carboplatin (5). To examine whether carboplatin could be efficiently encapsulated in a formulation, in which phosphatidylserine was replaced by another anionic lipid, we exchanged the phosphatidylserine for phosphatidylglycerol. We assayed the encapsulation efficiency and found that carboplatin was also efficiently encapsulated in phosphatidylcholine/phosphatidylglycerol bilayers (Table 2). However, encapsulation of carboplatin in phosphatidylcholine/phosphatidylglycerol (1:1) did not significantly enhance the cytotoxicity of carboplatin (Table 2), possibly due to other stability properties or to a difference in interaction with the cells compared with the phosphatidylcholine/phosphatidylserine formulation.

Lowering the phosphatidylserine content from 50% to 20% still yielded a pellet fraction with improved cytotoxicity (Table 2). The reduction of surface charge, achieved by incorporating less phosphatidylserine, can be beneficial for clinical application because it improves the stability of drug-lipid formulations in the bloodstream and decreases uptake by the reticuloendothelial system (34). In addition to surface charge, the size of the particles is important for clinical application because it affects the biodistribution and circulation time of the encapsulated drug after i.v. injection. Vesicles with a size between 100 and 200 nm accumulate in the angiogenic areas found in tumors (8, 34). We extruded the standard formulation through a 0.2-μm polycarbonate filter and assayed the effect on size, encapsulation efficiency, and cytotoxicity. Extrusion resulted in a decreased average size of 166 nm with a narrower size distribution (polydispersity index, 0.03) and values of encapsulation efficiency and cytotoxicity toward IGROV-1 cells comparable with those of the standard formulation (data not shown).

General Applicability of the Protocol to Platinum-Based Drugs

The model proposed for the formation of cisplatin nanocapsules postulates that cisplatin is concentrated in the residual fluid during freezing, giving rise to nanoprecipitates of dichloro-cisplatin covered by more soluble positively charged species. Subsequently, the negatively charged DOPC/DOPS vesicles interact with nanoprecipitates and reorganize to wrap them (22). The formulation and action of carboplatin nanocapsules reported here shows some remarkable parallels with the formation and action of cisplatin nanocapsules. We found that freeze-thawing and negatively charged lipids are required for formation and that the resulting lipid formulations contain an encapsulated drug concentration that exceeds the solubility limit of the drug. These observations suggest that carboplatin and cisplatin nanocapsules are formed via similar mechanisms despite the obvious differences in chemical structure, aquation rate, and solubility in water between the two drugs. Moreover, we showed that both formulations are taken up by cells through endocytosis and are extremely cytotoxic *in vitro* (21). This holds the promise that the same methodology could prove successful in the efficient encapsulation of other (platinum) drugs with limited water solubility and low lipophilicity and thereby improve the therapeutic index and profile of these drugs.

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