[6] Cisplatin Nanocapsules

By ANTON I. P. M. DE KROON, RUTGER W. H. M. STAFFHORST, BEN DE KRUIJFF, and KOERT N. J. BURGER

Abstract

Cisplatin nanocapsules represent a novel lipid formulation of the anticancer drug *cis*-diamminedichloroplatinum(II), in which nanoprecipitates of cisplatin are covered by a phospholipid bilayer coat consisting of an equimolar mixture of phosphatidylcholine and phosphatidylserine. Cisplatin nanocapsules are characterized by an unprecedented cisplatin-to-lipid molar ratio and exhibit strongly improved cytotoxicity against tumor cells *in vitro* compared with the free drug. Here, methods for preparing and characterizing cisplatin nanocapsules are reported.

Introduction

Cisplatin, *cis*-diamminedichloroplatinum(II), is an anticancer drug that is commonly used in the treatment of a variety of solid tumors, including genitourinary, head and neck, and lung (Lippert, 1999). The main cellular target of the drug appears to be the nuclear DNA, with which it forms stable adducts that interfere with transcription and replication, and trigger apoptosis, eventually resulting in the death of the cancer cell (Pinto and Lippard, 1985). The clinical use of cisplatin faces a number of serious problems. Due to its reactive nature, most of the drug is rapidly inactivated by binding to proteins upon entry in the blood by intravenous administration and never reaches the tumor in an active form (Howe-Grant and Lippard, 1980). Moreover, binding to proteins is considered a major cause of the many dose-limiting toxicities exhibited by cisplatin such as nephro-, oto-, and neurotoxicity (Calvert *et al.*, 1993; Hacker, 1991). Finally, the clinical utility of cisplatin is limited by the emergence of resistance in many tumor types (Perez, 1998).

One approach to try and circumvent these drawbacks of cisplatin is to encapsulate the drug in liposomes. Several liposomal formulations of cisplatin have been developed, the most recent being SPI-077, in which cisplatin is encapsulated in pegylated (''stealth'') liposomes consisting of hydrogenated soy phosphatidylcholine, cholesterol, and poly(ethyleneglycol) derivatized distearoylphosphatidylethanolamine (Newman *et al.*, 1999). Preclinical studies showed that compared with the free drug, SPI-077 exhibited

0076-6879/05 $35.00

improved stability, prolonged circulation time, increased antitumor effect, and reduced toxicity (Newman *et al.*, 1999; Vaage *et al.*, 1999). However, in Phase I/II studies, essentially no antitumor activity of SPI-077 was observed in patients (Harrington *et al.*, 2001; Kim *et al.*, 2001; Veal *et al.*, 2001).

A major problem of the conventional liposomal formulations of cisplatin, such as SPI-077, appears to be the limited bioavailability of the drug in the tumor (Bandak *et al.*, 1999; Meerum Terwogt *et al.*, 2002). A key factor is likely to be the low water solubility (7 m*M* at 37°) and low lipophilicity of cisplatin, which lead to liposomal formulations with low drug-to-lipid molar ratios (in the order of 0.02). Serendipitously, we discovered an alternative method to encapsulate cisplatin in a lipid formulation with superior efficiency (Burger *et al.*, 2002). Our method takes advantage of the limited solubility of the drug in water and results in cisplatin nanocapsules. Cisplatin nanocapsules are aggregates of cisplatin surrounded by a single lipid bilayer and have an unprecedented drug-to-lipid ratio and an unprecedented *in vitro* cytotoxicity.

Principle

Nanocapsules are prepared by repeatedly freezing and thawing a concentrated aqueous solution of cisplatin in the presence of negatively charged phospholipids. The preparation of cisplatin nanocapsules was shown to depend critically on the presence of negatively charged phospholipids and on the presence of positively charged aquo species of cisplatin, pointing to an essential role for electrostatic interaction in the mechanism of formation. A solution of cisplatin in water, in the absence of added chloride, contains a mixture of the neutral dichloride and dihydroxo species of cisplatin with low solubility in water and positively charged aqua species of cisplatin with a much higher solubility (Lippert, 1999). In our current model (Fig. 1) for the mechanism of nanocapsule formation (Burger *et al.*, 2002), cisplatin is concentrated in the residual fluid during freezing, and it forms small aggregates when the solubility limit of the dichloro species is exceeded. Subsequently, the aggregates of the dichloro species of cisplatin are covered by the positively charged aqua species, which have a higher solubility limit. The negatively charged membranes interact with the positively charged cisplatin aggregates and then reorganize to wrap the aggregates in a phospholipid bilayer coat. The resulting nanocapsules do not redissolve upon thawing.

Method

Cisplatin (Sigma, St. Louis, MO) is dissolved in MilliQ water to a concentration of 5 m*M*, which is facilitated by incubating at 55° for

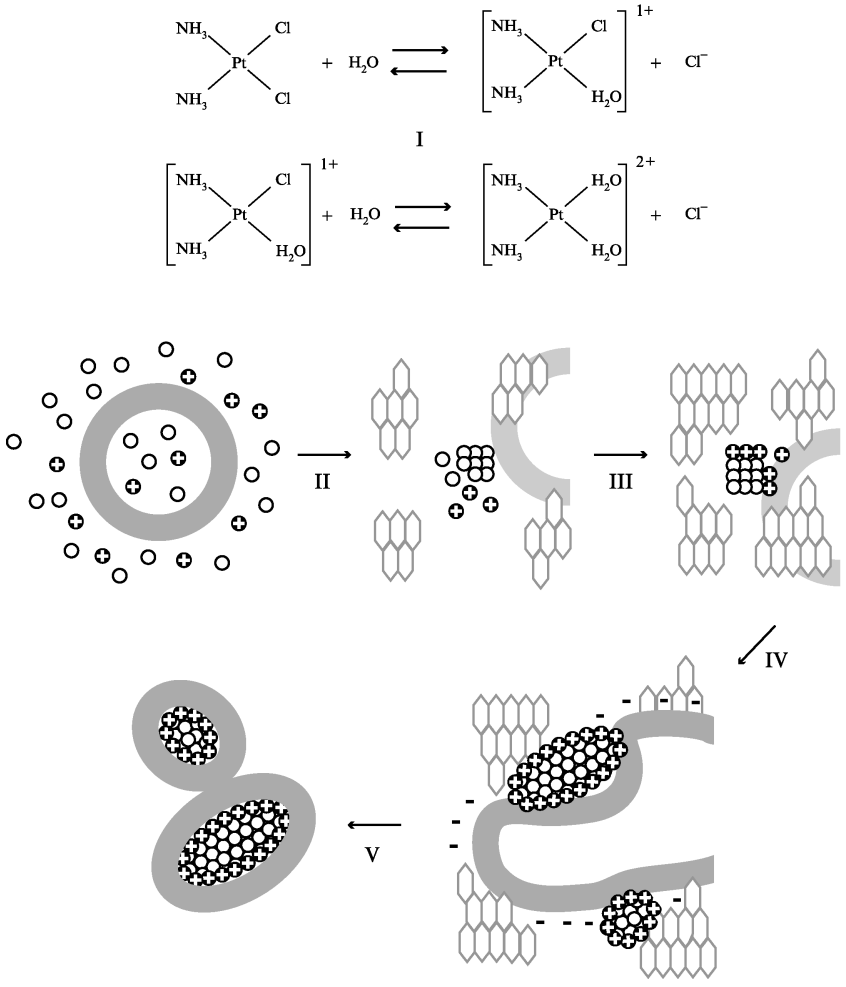

FIG. 1. Model of cisplatin nanocapsule formation. Partial hydrolysis of cisplatin in water yields positively charged aqua species (I). When a suspension of negatively charged liposomes is frozen in the presence of neutral (white spheres) and positively charged species (+ marked black spheres) of cisplatin, the formation of ice crystals drives the aggregation of the neutral species (II). As the ice crystals grow and the fluid phase is further concentrated, the positively charged species coaggregate (III), eventually covering the neutral aggregates, which then interact with the negatively charged liposomes (IV). The membranes reorganize to surround the cisplatin aggregates, and upon thawing, nanocapsules have been formed (V).

30 min. The solution is incubated overnight in the dark at 37° to ensure full equilibration.

Stock solutions of 1,2-dioleoyl-*sn*-glycer-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoserine (DOPS), obtained from Avanti Polar Lipids, Inc. (Alabaster, AL), are prepared in chloroform (concentration ~5 m*M*). The exact concentrations are determined by phosphate analysis (Rouser *et al.*, 1970). Aliquots corresponding to 0.6 μmol of each phospholipid are mixed, the solvent is removed by rotary evaporation, and the lipid film is further dried under vacuum overnight.

The dry lipid film is hydrated by adding 1.2 ml of the 5 m*M* cisplatin solution and incubation for 15 min at 37°. After brief homogenization in a vortex mixer, the dispersion is transferred to a glass tube and subjected to 10 freeze–thaw cycles using ethanol/dry-ice (−70°) and a waterbath (37°).

The resulting colloidal solution is transferred to microfuge tubes and centrifuged for 4 min at 470*g* (2100 rpm) in an Eppendorf centrifuge to collect the nanocapsules. After removal of the supernatant, the fluffy white layer on top of the yellow pellet, corresponding to large liposomes, is removed by a micropipette. The yellow pellet containing the cisplatin nanocapsules is resuspended in 1 ml water and centrifuged as above to wash away nonencapsulated cisplatin. Upon resuspending the final pellet in 0.5 ml water, the nanocapsules are stored at 4° until use.

Alternatively, the nanocapsules are purified from contaminating liposomes by density gradient centrifugation (Burger *et al.*, 2002). Briefly, the dispersion obtained after the freeze–thaw cycles is loaded on top of a step gradient consisting of 1 ml of each 1.8 *M*, 0.6 *M*, and 0.2 *M* sucrose in 10 m*M* PIPES-NaOH, 1 m*M* EGTA, pH 7.4. After centrifugation at 4° for 30 min at 400,000*g* (SW60 rotor, Beckman), the pellet fraction corresponding to the nanocapsules is collected and washed as above.

Remarks

Instead of DOPS, the negatively charged phospholipids dioleoylphoshatidylglycerol (DOPG) and dioleoylphosphatidic acid (DOPA) can also be used to prepare cisplatin nanocapsules. Likewise, DOPC can be replaced by dioleoylphosphatidylethanolamine (DOPE) or sphingomyelin (Burger *et al.*, 2002).

The method is sensitive to high chloride concentrations and alkaline pH, because these conditions prevent the formation of the positively charged aqua species (Lippert, 1999).

Instead of hydrating a lipid film with 5 m*M* cisplatin, it is also possible to add the cisplatin solution to preformed DOPC/DOPS liposomes and then start the freeze–thaw cycles.

The cisplatin nanocapsules can be stored after lyophilization and retain cisplatin upon rehydration.

Characterization

Encapsulation Efficiency

The phospholipid content of the nanocapsules is determined by phosphate analysis (Rouser *et al*., 1970). The cisplatin content of the nanocapsules is assessed by flameless atomic absorption spectrometry (NFAAS) using K_2PtCl_2 (Sigma) as a standard (Burger *et al*., 1999; van Warmerdam *et al*., 1995). For this purpose, an aliquot of the cisplatin nanocapsule suspension is dissolved and diluted in 0.05% (w/v) Triton X-100, 0.05% (w/v) HNO_3 to a Pt concentration in the calibration range of the spectrometer (e.g., 2–10 ng K_2PtCl_2 per 30 μl sample volume for a Varian SpectrAA-400 Zeeman spectrometer).

Analysis of the cisplatin nanocapsules prepared as above typically yields a Pt/phosphate molar ratio of 11 ± 1. Based on the size of the nanocapsules (see below), this number is estimated to correspond to an internal cisplatin concentration exceeding 5 *M*, which is far beyond the solubility limit of cisplatin and consistent with the quasicrystalline structure of the encapsulated cisplatin (Burger *et al*., 2002). The method encapsulates cisplatin with an efficiency of approximately 30%.

Shape and Size

Analysis by negative stain electron microscopy reveals bean-shaped particles consisting of an electron-dense core surrounded by a bright layer (excluding stain), corresponding to the bilayer coat (Fig. 2) (Burger *et al*., 2002). The nanocapsules have a heterogeneous size distribution, with 75% of the population having a length between 50 and 250 nm and a width of around 50 nm (Fig. 2). Size analysis by dynamic light scattering yields consistent results (Burger *et al*., 2002). Smaller size nanocapsules with a narrower size distribution have been obtained by high-pressure extrusion through polycarbonate filters with a 200-nm pore size (Burger *et al*., 2002; Hope *et al*., 1986).

Cytotoxicity

The cytotoxicity of the cisplatin nanocapsules toward the human ovarian carcinoma IGROV-1 cell line has been compared with that of free cisplatin. The IC_{50} value (the drug concentration at which cell growth is inhibited by 50%) of cisplatin administered as nanocapsules is two orders

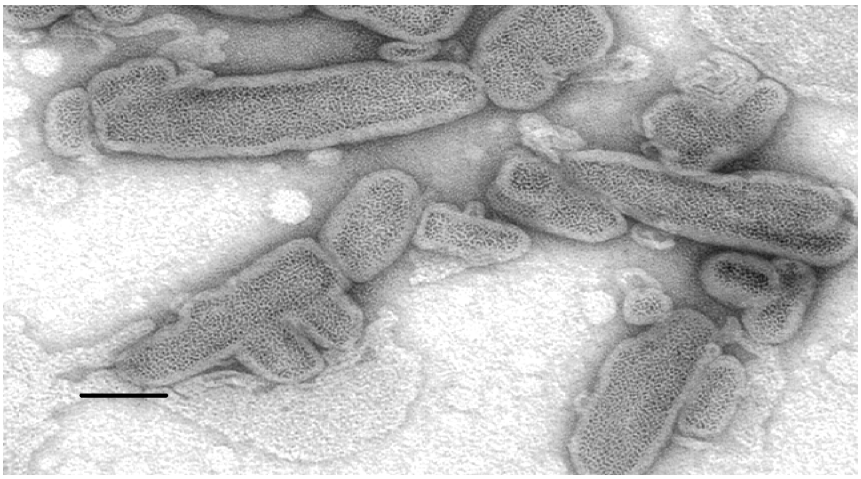

FIG. 2. Electron micrograph of cisplatin nanocapsules visualized by negative staining. A dilute suspension of nanocapsules was transferred to a carbon-Formvar–coated grid, dried, and stained with 4% (w/v) uranyl acetate for 45 s. Scale bar, 50 nm. Figure reproduced from Velinova *et al.* (2004) with permission.

of magnitude smaller than that of the free drug (Burger *et al.*, 2002). The higher cytotoxicity is explained by the reduced inactivation of the drug, due to the lipid coat sequestering it from reaction with substrates in the extracellular environment. Upon binding to the cell surface or endocytotic uptake of the nanocapsules, the coat is destabilized, and after membrane passage, cisplatin exerts its cytotoxic effect.

Conclusion

The cisplatin nanocapsules represent a new lipid formulation of cisplatin, distinct from conventional liposomal formulations. The distinctive feature is that the drug is present as aggregates surrounded by a bilayer. This results in a drug-to-lipid ratio that is two to three orders of magnitude higher than that of liposomal formulations (Newman *et al.*, 1999; Peleg-Shulman *et al.*, 2001) and probably accounts for the characteristic bean shape of the cisplatin nanocapsules. The increased encapsulation efficiency of cisplatin in nanocapsules is expected to increase the bioavailability of the drug and thus improve the therapeutic index compared with liposomal formulations of cisplatin. The method for preparing cisplatin nanocapsules may be applicable to other compounds that, due to their limited solubility in water and low lipophilicity, are not amenable to encapsulation in conventional liposomes.

Acknowledgments

The authors thank W. Mulder and M. J. Velinova for experimental support. The financial support by the Dutch Cancer Society (project UU2001-2493) is gratefully acknowledged.

Note added in proof: The molecular architecture of the cisplatin nanocapsules was recently solved (Chupin, V., de Kroon, A. I. P. M., and de Kruÿff, B. (2004). Molecular architecture of nanocapsules, bilayer-enclosed solid particles of cisplatin. *J. Am. Chem. Soc.* **126,** 13816–13821).

References

Bandak, S., Goren, D., Horowitz, A., Tzemach, D., and Gabizon, A. (1999). Pharmacological studies of cisplatin encapsulated in long-circulating liposomes in mouse tumor models. *Anticancer Drugs* **10,** 911–920.

Burger, K. N. J., Staffhorst, R. W. H. M., and de Kruijff, B. (1999). Interaction of the anticancer drug cisplatin with phosphatidylserine in intact and semi-intact cells. *Biochim. Biophys. Acta* **1419,** 43–54.

Burger, K. N. J., Staffhorst, R. W. H. M., de Vijlder, H. C., Velinova, M. J., Bomans, P. H., Frederik, P. M., and de Kruijff, B. (2002). Nanocapsules: Lipid-coated aggregates of cisplatin with high cytotoxicity. *Nat. Med.* **8,** 81–84.

Calvert, H., Judson, I., and van der Vijgh, W. J. (1993). Platinum complexes in cancer medicine: Pharmacokinetics and pharmacodynamics in relation to toxicity and therapeutic activity. *Cancer Surv.* **17,** 189–217.

Hacker, M. P. (1991). *In* ''The Toxicity of Anticancer Drugs'' (G. Powis and M. P. Hacker, eds.), p. 82. McGraw-Hill, New York.

Harrington, K. J., Lewanski, C. R., Northcote, A. D., Whittaker, J., Welbank, H., Vile, R. G., Peters, A. M., and Stewart, J. S. (2001). Phase I-II study of pegylated liposomal cisplatin (SPI-077) in patients with inoperable head and neck cancer. *Ann. Oncol.* **12,** 493–496.

Hope, M. J., Bally, M. B., Mayer, L. D., Janoff, A. S., and Cullis, P. R. (1986). Generation of multilamellar and unilamellar phospholipid vesicles. *Chem. Phys. Lipids* **40,** 89–107.

Howe-Grant, M. E., and Lippard, S. J. (1980). Aqueous platinum(II) chemistry; binding to biological molecules. *In* ''Metal Ions in Biological Systems'' (H. Sigel, ed.), Vol. XI, pp. 63–125. Marcel Dekker, New York.

Kim, E. S., Lu, C., Khuri, F. R., Tonda, M., Glisson, B. S., Liu, D., Jung, M., Hong, W. K., and Herbst, R. S. (2001). A phase II study of STEALTH cisplatin (SPI-77) in patients with advanced non-small cell lung cancer. *Lung Cancer* **34,** 427–432.

Lippert, B. (1999). ''Cisplatin: Chemistry and Biochemistry of a Leading Anticancer Drug.'' Wiley-VCH, New York.

Meerum Terwogt, J. M., Groenewegen, G., Pluim, D., Maliepaard, M., Tibben, M. M., Huisman, A., ten Bokkel Huinink, W. W., Schot, M., Welbank, H., Voest, E. E., Beijnen, J. H., and Schellens, J. H. M. (2002). Phase I and pharmacokinetic study of SPI-77, a liposomal encapsulated dosage form of cisplatin. *Cancer Chemother. Pharmacol.* **49,** 201–210.

Newman, M. S., Colbern, G. T., Working, P. K., Engbers, C., and Amantea, M. A. (1999). Comparative pharmacokinetics, tissue distribution, and therapeutic effectiveness of cisplatin encapsulated in long-circulating, pegylated liposomes (SPI-077) in tumor-bearing mice. *Cancer Chemother. Pharmacol.* **43,** 1–7.

Peleg-Shulman, T., Gibson, D., Cohen, R., Abra, R., and Barenholz, Y. (2001). Characterization of sterically stabilized cisplatin liposomes by nuclear magnetic resonance. *Biochim. Biophys. Acta* **1510,** 278–291.

Perez, R. P. (1998). Cellular and molecular determinants of cisplatin resistance. *Eur. J. Cancer* **34,** 1535–1542.

Pinto, A. L., and Lippard, S. J. (1985). Binding of the antitumor drug cis-diamminedichloroplatinum(II) (cisplatin) to DNA. *Biochim. Biophys. Acta* **780,** 167–180.

Rouser, G., Fleischer, S., and Yamamoto, A. (1970). Two-dimensional thin-layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids* **5,** 494–496.

Vaage, J., Donovan, D., Wipff, E., Abra, R., Colbern, G., Uster, P., and Working, P. (1999). Therapy of a xenografted human colonic carcinoma using cisplatin or doxorubicin encapsulated in long-circulating pegylated stealth liposomes. *Int. J. Cancer* **80,** 134–137.

van Warmerdam, L. J. C., van Tellingen, O., Maes, R. A. A., and Beijnen, J. H. (1995). Validated method for the determination of carboplatin in biological-fluids by Zeeman atomic-absorption spectrometry. *Fresenius J. Anal. Chem.* **351,** 777–781.

Veal, G. J., Griffin, M. J., Price, E., Parry, A., Dick, G. S., Little, M. A., Yule, S. M., Morland, B., Estlin, E. J., Hale, J. P., Pearson, A. D., Welbank, H., and Boddy, A. V. (2001). A phase I study in paediatric patients to evaluate the safety and pharmacokinetics of SPI-77, a liposome encapsulated formulation of cisplatin. *Br. J. Cancer* **84,** 1029–1035.

Velinova, M. J., Steffhorst, R. W. H. M., Mulder, W. J. M., Dries, A. J., Jensen, B. A. J., de Kruÿff, B., and de Kroon, A. I. P. M. (2004). Preparation and stability of lipid-coated nanocapsules of cisplatin: Anionic phospholipid specificity. *Biochim. Biophys. Acta* **1663,** 135–142.

[7] Liposomal Cytokines in the Treatment of Infectious Diseases and Cancer

By TIMO L. M. TEN HAGEN

Abstract

Despite of the demonstrated activity of cytokines *in vitro*, their use in the clinical setting is often disappointing. Cytokine-related toxicity seriously limits optimal use *in vivo*. In addition, rapid degradation and excretion, neutralization and binding to receptors, or metabolization of the molecule results in a short half-life in serum when injected intravenously. As the dose–response curve of cytokines is relatively steep, outcome greatly benefits from improved delivery and bioavailability. One way to improve the pharmacokinetics of cytokines after systemic application is encapsulation in liposomes. An advantage of liposomes is that the encapsulated drug is protected from (rapid) degradation and excretion, and it eliminates the binding to neutralizing antibodies or (soluble) receptors. Moreover, liposomes can be tailored in such a way that they exhibit favorable pharmacokinetics, i.e., increased serum half-life and improved targeting to tissues or cells of interest. In this chapter, the use of liposomal cytokines in the treatment of cancer and infectious disease is discussed.

METHODS IN ENZYMOLOGY, VOL. 391
0076-6879/05 $35.00