Toxicity and Antitumor Activity of cis-Bis-cyclopentenecarboxylato-1,2-diaminocyclohexane Platinum(II) Encapsulated in Multilamellar Vesicles


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ABSTRACT

The potential of multilamellar vesicles (MLVs) as carriers of cis-bis-cyclopentenecarboxylato-1,2-diaminocyclohexane platinum(II) (CPDP), a lipophilic cisplatin derivative, was assessed. MLVs composed of dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylglycerol (DMPG), and cholesterol at different molar ratios were tested. The MLV-CPDP preparation with the highest antitumor activity against L1210 leukemia in vivo was DMPGDMPG 7:3-CPDP. The encapsulation efficiency of this preparation was 66 ± 4% (SD); the stability in 0.9% NaCl solution at 4°C was 89% at 14 days and 93% 18 h after incubation in human AB serum at 37°C. The toxicities of DMPGDMPG 7:3-CPDP and free CPDP (suspended in hydroxypropyl cellulose) administered i.p. were similar (50% lethal dose = 75 versus 91 mg/kg; blood urea nitrogen values 96 h after the administration of the 50% lethal dose = 32.0 versus 34.4 mg/dl). The mean %/T (median survival time of treated mice + median survival time of control mice) × 100% obtained after a single i.p. injection of the optimal dose of each preparation tested was 215 (range 200 to 232) for DMPGDMPG 7:3-CPDP, 175 (range 158 to 209) for DMPG-CPDP, 162 (range 136 to 179) for free CPDP, and 178 (range 169 to 189) for cisplatin. Using a multiple i.p. injection schedule (injections on Days 1, 5, and 9), DMPGDMPG 7:3-CPDP was more active than free CPDP and cisplatin (%/T/C: 403, 284, and 253%, respectively). DMPGDMPG 7:3-CPDP is less toxic and more active against L1210 leukemia in vivo than is cisplatin. The encapsulation of CPDP in MLVs composed of DMPC:DMPG 7:3 provides an adequate vehicle for the administration of this lipophilic compound and enhances its antitumor activity against L1210 leukemia.

INTRODUCTION

Liposomes are lipid vesicles that form spontaneously upon addition of an aqueous solution to a dry lipid film (1). Liposomes can be used as drug carriers for lipophilic or hydrophilic drugs entrapped in their respective lipophilic or hydrophilic compartment. MLVs(1) are multilayer lipid vesicles that are particularly suited for carrying lipophilic drugs since their lipophilic compartment is larger than their hydrophilic compartment. When injected i.v. in animals (2, 3) and humans (4), MLVs concentrate in the liver, spleen, and other organs rich in reticuloendothelial cells.

Liposomes have been previously used to deliver chemotherapeutic agents (1), immunomodulators, and antifungal agents in vitro (5, 6) and in vivo (7–10) in animals and humans (11).

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5 The abbreviations used are: MLV, multilamellar liposome; DMPC, dimyristoyl phosphatidylcholine; DMPG, dimyristoyl phosphatidylglycerol; CPDP, cis-bis-cyclopentenecarboxylato-1,2-diaminocyclohexane platinum(II); LD(50), liposomal-platinum preparation; LD(15), LD(50), or LD(100), the dose lethal to 10, 50, or 90%, respectively, of the animals tested; BUN, blood urea nitrogen; IC(50), the concentration that achieves a 50% inhibition of growth; ST/C, (median survival time of treated mice divided by the median survival time of control mice) × 100; cisplatin, cis-diaminedichloroplatinum(II).

Recent studies show that liposomes can reduce certain types of drug-related toxicity (such as doxorubicin cardiotoxicity) (12–15) and may increase the antitumor activity as a result of a slow-release mechanism (in the case of 1,2-d-arabinofuranosylcytosine) (16, 17), a higher drug uptake by tumor cells, or a more favorable drug distribution throughout organs (18, 19). In spite of these promising results, the clinical application of antitumor agents encapsulated in liposomes has been delayed mainly due to formulation, stability, and large-scale production problems.

Cisplatin is a highly effective drug in the treatment of several neoplastic diseases in humans (20). However, its use is limited by severe systemic toxicity, particularly nephrotoxicity and neurotoxicity (21). In an attempt to modify the therapeutic index of cisplatin, analogues which are less toxic and non-cross-resistant have been synthesized during the last decade. However, the development of some promising analogues has been hampered by their low hydrosolubility, which decreases their potential for clinical use (22). Cisplatin has been previously encapsulated in MLVs but with a very low encapsulation efficiency (7.4%) and poor stability (75% at 48 h in 0.9% NaCl solution) (23). In order to improve these characteristics, we explored the possibility that a water-insoluble cisplatin analogue might be a better candidate for liposome encapsulation. Using this approach, we have developed a liposomal-platinum preparation which has a high encapsulation efficiency, good stability, is devoid of nephrotoxicity, and has in vivo antitumor activity against L1210 leukemia at least comparable with that of cisplatin.

MATERIALS AND METHODS

Chemicals and Lipids. K2PtCl4 was purchased from AESAR (Johnson Matthey, Inc., Seabrook, NH). Cyclopentenecarboxylic acid was purchased from Pfaltz and Bauer, Inc., Stamford, CT; 1,2-diaminocyclohexane was purchased from Aldrich Chemical Co., Milwaukee, WI. Elemental analysis on the platinum complex was performed by Integral Microanalytical Laboratories, Inc., Raleigh, NC. The infrared spectrum of the complex (as KBr pellet) was measured in the range of 600 to 4000 cm⁻¹ using a Nicolet 6000 Fourier transform infrared spectrophotometer.

Chromatographically pure (thin-layer chromatography) DMPC and DMPG used in this study were obtained from Avanti Polar Lipids (Birmingham, AL). Cholesterol was purchased from Sigma Chemical Co. (St. Louis, MO).

Synthesis of CPDP. CPDP is the cisplatin lipophilic analogue used as a prototype to develop the liposomal-platinum preparation (Fig. 1). CPDP was synthesized as reported previously (24) using the following multistep procedure: 0.96 g of 1,2-diaminocyclohexane was added to a filtered aqueous solution of K2PtCl4 (3.5 g in 50 ml of H2O) and the mixture was stirred for 6 to 8 h at room temperature. The yellow solid was removed by filtration and washed with H2O, methanol, and acetone. After the final product was dried under vacuum, the yield was calculated to be 56%. Subsequently, 1.0 g of cis-bis-dichloro-1,2-diaminocyclohexane platinum(II) was suspended in 20 ml of H2O and an aqueous solution of K2PtCl4 (3.5 g in 50 ml of H2O) and the mixture was stirred for 6 to 8 h at room temperature. The yellow solid was removed by filtration and washed with H2O, methanol, and acetone. After the final product was dried under vacuum, the yield was calculated to be 56%. Subsequently, 1.0 g of cis-bis-dichloro-1,2-diaminocyclohexane platinum(II) was suspended in 20 ml of H2O and an aqueous solution of Ag2SO4 (0.75 g in 150 ml H2O) was added to obtain water-
soluble sulfato-1,2-diaminocyclohexane platinum(II). The reaction mixture was stirred in the dark for 24 h and the precipitated AgCl was removed by filtration. The yellow solution was evaporated to dryness at 45–50°C under reduced pressure and the yellow-brown product was further dried over P₂O₅ under vacuum. The yield of sulfato-1,2-diaminocyclohexane platinum(II) 90%. Finally, 0.423 g (1 mmol) of sulfato-1,2-diaminocyclohexane platinum(II) dissolved in 100 ml of H₂O was added to barium cyclopentenecarboxylate [prepared in situ by adding 0.3 g of BaOH.8 H₂O to 0.226 g (2 mmol) of cyclopentenecarboxylic acid in H₂O], and the reaction mixture was stirred for 30 min at room temperature. The BaSO₄ precipitate was filtered off and the yellow filtrate was evaporated to dryness at 45°C under reduced pressure using a rotary evaporator. A solid was obtained, which was purified from methanol. The product was finally dried under vacuum. The yield was 70%.

\[
\text{Encapsulation efficiency at } X \text{h} = \frac{\text{Total platinum in the initial liposome suspension}}{\text{Total CPDP initially added}} \times 100
\]

The encapsulation efficiency values used in the stability determinations were obtained by measuring CPDP in the supernatants by UV spectrophotometry (for the preparations in 0.9% NaCl solution) or platinum by X-ray fluorescence (for the preparations in human AB serum). The stability of L-CPDP in 0.9% NaCl solution was determined up to 14 days after the initial preparation. In addition, the L-CPDP preparations were observed microscopically on Day 14 to check the morphology of the vesicles. The stability in human AB serum was determined up to 18 h after incubation of 0.5 ml of liposome suspension in 0.5 ml human AB serum.

Toxicity Studies. Toxicology studies were carried out in 6- to 8-week-old CD1 Swiss mice weighing 22 to 25 g purchased from The University of Texas Science Park (Bastrop, TX). Groups of 8 mice each were given free CPDP suspended in hydroxypropyl cellulose, DMPG, DMPG-CPDP, DMPC:DMPG 7:3, and DMPC:DMPG 7:3-CPDP i.p. in volumes ranging between 0.1 and 0.3 ml. DMPC:DMPG 7:3-CPDP was also administered i.v. in one single or three daily injections. The clinical behavior and survival times of the mice were monitored on a daily basis. The LD₅₀, LD₇₀, and LD₉₀ were calculated considering the deaths occurring up to 14 days after injection.

Nephrotoxicity. BUN was determined in samples obtained from the retroorbital plexus of CD1 Swiss mice weighing 22 to 25 g 96 h after a single i.p. injection of cisplatin, CPDP in hydroxypropyl cellulose, DMPG-CPDP, and DMPC:DMPG 7:3-CPDP at doses corresponding to the previously determined LD₅₀. All L-CPDP preparations tested for toxicity were prepared under sterile conditions on the same day of the experiment.

**In Vivo Antitumor Activity against L1210 Cells.** L1210 leukemic cells were grown in a suspension culture in McCoy's 5A medium (Gibco Laboratories, Grand Island, NY) supplemented with 10% horse serum, glutamine, streptomycin, and penicillin at 37°C and 95% relative humidity in a 5% CO₂ atmosphere. Four ml of cell suspension were added to culture tubes and the appropriate concentration of CPDP in hydroxypropyl cellulose or L-CPDP was added (final concentration, 0.01 to 10 μg/ml). After 96 h, the cell concentrations of control and experimental cultures were calculated with a Coulter Counter (Coulter Electronics, Hialeah, FL) and the inhibition percentage was calculated. The following preparations were tested: CPDP in hydroxypropyl cellulose; DMPG; DMPC:DMPG 7:3; DMPG-CPDP; and DMPC:DMPG 7:3-CPDP. Results were expressed as IC₅₀. Results for empty liposomes were expressed as the amount of CPDP that would have been encapsulated at the IC₅₀ concentration.

**In Vivo Antitumor Activity against L1210 Mouse Leukemia.** The *in vivo* antitumor activities of cisplatin, CPDP in hydroxypropyl cellulose, DMPG, DMPG-CPDP, DMPC:DMPG 7:3, and DMPC:DMPG 7:3-CPDP were tested in an L1210-C57BL x DBA/2 F₁ (hereafter called BD2F₁) mouse model. BD2F₁ mice were purchased from Charles River (Wilmington, MA). Groups of 6 to 8 mice weighing 18 to 22 g were inoculated i.p. with 10⁶ L1210 leukemia cells on Day 0. L1210 cells were kept in DBA/2 mice with weekly cell passages between the different experiments. All L-CPDP preparations were injected i.p. in volumes of 0.1 to 0.3 ml 24 h after tumor inoculation. Two different schedules of administration were used: a single injection on Day 1 or 3 separate injections on Days 1, 5, and 9. The doses of cisplatin used were the ones that had resulted in a maximum antitumor activity in previous experiments. The doses of CPDP, DMPG-CPDP, and DMPC:DMPG 7:3-CPDP used ranged from 3.125 to 50 mg/kg (approximate LD₅₀). Clinical behavior and survival times of the mice were monitored until all animals had died. Results were expressed as %T/C and number of long-term survivors. Mice living more than 30 days and more than 60 days were considered to be long-term survivors for the single- and multiple-injection schedules, respectively. All L-CPDP preparations tested for antitumor activity were prepared under sterile conditions on the same day of the experiment.

**Fig. 1. Chemical structure of cis-bis-cyclopentenecarboxylato-1,2-diaminocyclohexane platinum(II).** PT, Pt.
RESULTS

Encapsulation Efficiency of L-CPDP Preparations of Different Lipid Composition. In the initial experiments using 50-ml flasks, the encapsulation efficiency of the different L-CPDP preparations varied from 27% for DMPC-CPDP to 55% for DMPG-CPDP (Table 1). The encapsulation efficiency of the DMPC:DMPG-CPDP preparations ranged between 37 and 48%. Addition of cholesterol did not result in an increased encapsulation efficiency. In subsequent experiments, using 250-ml flasks, the encapsulation efficiency of DMPC:DMPG 7:3-CPDP was markedly increased (66 ± 4%) (SD was the mean of 12 different preparations). Among all the L-CPDP preparations initially tested, DMPG-CPDP and DMPC:DMPG 7:3-CPDP were selected for further studies.

Stability of L-CPDP. The stability of DMPG-CPDP in 0.9% NaCl solution was 95% at 72 h versus 91% for DMPC:DMPG 7:3-CPDP. When both preparations were incubated for 18 h in human AB serum at 37°C, the stability was 97% for DMPC-CPDP and 93% for DMPC:DMPG 7:3-CPDP. Subsequently, DMPC-CPDP and DMPC:DMPG 7:3-CPDP were incubated for 14 days in 0.9% NaCl solution at 4°C. In the DMPC:DMPG 7:3-CPDP preparation, no morphological evidence of vesicle destruction was observed by light microscopy, and 89% of the drug was still entrapped in the liposomes on Day 14. The DMPG-CPDP preparation, on the other hand, showed significant vesicle disruption and formation of insoluble clumps.

Toxicology Studies. CPDP in hydroxypropyl cellulose, DMPG-CPDP, and DMPC:DMPG 7:3-CPDP had similar LD50 dose levels when given in a single i.p. injection (91, 86, and 75 mg/kg, respectively) (Table 2). The amount of CPDP for 14 days in 0.9% NaCl solution at 4°C. In the DMPC:DMPG 7:3-CPDP preparation, no morphological evidence of vesicle destruction was observed by light microscopy, and 89% of the drug was still entrapped in the liposomes on Day 14. The DMPG-CPDP preparation, on the other hand, showed significant vesicle disruption and formation of insoluble clumps.

In Vitro Antitumor Activity against L1210 Leukemia. The IC50 of CPDP in hydroxypropyl cellulose was 1.3 μg/ml (Table 4). Of the two L-CPDP preparations tested, DMPC-CPDP was slightly more active than DMPC:DMPG 7:3-CPDP (mean IC50 of three experiments, 0.7 μg/ml for DMPC-CPDP versus 1.6 μg/ml for DMPC:DMPG 7:3-CPDP). Empty liposomes composed of DMPG had an IC50 within the range of activity (3.7 μg/ml) and the IC50 for empty liposomes composed of DMPG:DMPG 7:3 was >10 μg/ml (Table 4).

In Vivo Antitumor Activity against L1210 Leukemia. In the first set of experiments, the effect of a single i.p. dose of cisplatin, CPDP in hydroxypropyl cellulose, DMPG-CPDP, and DMPC:DMPG 7:3-CPDP in the treatment of L1210 leukemia was tested. Mice treated with CPDP in hydroxypropyl cellulose at doses of 25 and 12.5 mg/kg had a %T/C comparable with the %T/C of those treated with cisplatin, 10 mg/kg (162 versus 178, mean of three experiments) (Table 5). DMPC-CPDP at doses of 12.5, 6.25, and 3.125 mg/kg had an antitumor activity comparable with that of CPDP in hydroxypropyl cellulose and cisplatin (mean %T/C = 175 for 12.5 mg/kg, 158 for 6.25 mg/kg, and 163 for 3.125 mg/kg) (Table 5). DMPC-CPDP doses of 25 mg/kg or more were toxic for L1210 leukemia-bearing BD2F1 mice. Mice treated with DMPC:DMPG 7:3-CPDP at doses of 25, 12.5 and 6.25 mg/kg had a similar or slightly higher %T/C than those obtained with cisplatin, CPDP in hydroxypropyl cellulose, or DMPG-CPDP (mean %T/C = 215 for 25 mg/kg, 178 for 12.5 mg/kg, and 200 for 6.25 mg/kg) (Table 5). DMPC-CPDP at a dose of 50 mg/kg was toxic for L1210 leukemia-bearing BD2F1 mice. Empty liposomes composed of DMPC and DMPG-CPDP:7:3 at doses equivalent to the optimal ones of loaded vesicles did not show antitumor activity (%T/C = 105 for DMPG and 93 for DMPC:DMPG 7:3). Long-term survivors (on Day 30) were seen in the groups treated with cisplatin, DMPC-CPDP, and DMPC:DMPG 7:3-CPDP (Table 5).

In the experiments using a multiple-dose schedule, the high
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Table 5 In vivo antitumor activity against mouse L1210 leukemia of cisplatin, free CPDP, and L-CPDP administered i.p. (Day 1)

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>%T/C (no. of survivors on Day 30)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1*</td>
</tr>
<tr>
<td>Cisplatin</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>167</td>
</tr>
<tr>
<td>CPDP in hydroxypropyl cellulose</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>12.5</td>
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<tr>
<td>DMPG-CPDP</td>
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<tr>
<td>25</td>
<td>12.5</td>
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<tr>
<td>6.25</td>
<td>158</td>
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<tr>
<td>3.12</td>
<td>163 (2/6)</td>
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<tr>
<td>DMPG:DMPG 7:3-CPDP</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>12.5</td>
</tr>
<tr>
<td>6.25</td>
<td>167 (1/6)</td>
</tr>
</tbody>
</table>

* Median survival time of control mice = 7.5 days.

Table 6 In vivo antitumor activity against mouse L1210 leukemia of cisplatin, free CPDP, and L-CPDP administered i.p. (Days 1, 5, and 9)

All preparations were injected i.p. in volumes of 0.1 to 0.3 ml 24 h, 5 days, and 9 days after tumor inoculation.

<table>
<thead>
<tr>
<th>Preparations</th>
<th>Dose (mg/kg × 3)</th>
<th>%T/C (no. of survivors on Day 60)</th>
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<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
<td>Experiment 2</td>
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<tr>
<td>Cisplatin</td>
<td>7.5</td>
<td>253</td>
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<td></td>
<td></td>
<td>200</td>
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<tr>
<td>CPDP in hydroxypropyl cellulose</td>
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<tr>
<td>25</td>
<td>12.5</td>
<td>284</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
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<td>DMPG-CPDP</td>
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<td>6.25</td>
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<td></td>
<td>3.12</td>
<td>168</td>
</tr>
<tr>
<td></td>
<td>1.56</td>
<td>115</td>
</tr>
<tr>
<td>DMPG:DMPG 7:3-CPDP</td>
<td></td>
<td>403 (1/6)</td>
</tr>
<tr>
<td>12.5</td>
<td>6.25</td>
<td>253</td>
</tr>
<tr>
<td></td>
<td>3.125</td>
<td>210 (1/6)</td>
</tr>
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</table>

The encapsulation of CPDP in MLVs composed of DMPC:DMPG 7:3 provided an adequate vehicle for the administration of a water-insoluble cisplatin analogue that otherwise could not be used for i.v. administration, and resulted in an enhancement of its antitumor activity when a multiple-injection schedule was used. Compared with cisplatin, DMPC:DMPG 7:3-CPDP was less toxic, with an LD₅₀ approximately four times greater and no BUN elevation at the LD₅₀ dose level, and also more active against L1210 leukemia, the net result being an improved therapeutic index. The antitumor activity was studied in the L1210 leukemia model because it is the standard screening system used for cisplatin analogues. However, it is probably not the best model to test the potential advantages of antitumor drug encapsulation in liposomes since no tumor targeting is involved. In spite of this, DMPC:DMPG 7:3-CPDP was as active as cisplatin at molar doses two to three times lower than the optimal dose of cisplatin both in the single- and triple-injection schedules used.

Encapsulation of cisplatin in liposomes has been attempted previously with little success (23). A high encapsulation efficiency and stability are prerequisites for potential clinical application of any liposomal preparation. Our study has shown that by using a lipophilic cisplatin analogue and a mixture of two physiological phospholipids (DMPC and DMPG), a high encapsulation efficiency and stability can be obtained with full preservation of the favorable toxicity properties of the analogue used and even some enhancement of the antitumor activity. Still, we expect to be able to optimize the encapsulation efficiency and stability by using other lipophilic analogues specifically designed for liposome encapsulation.

Although both the decreased renal toxicity and increased LD₅₀ of L-CPDP were related to intrinsic properties of the analogue used and not to liposome encapsulation, liposome encapsulation of platinum complexes may by itself modify their therapeutic index by altering well-known platinum toxicities such as emesis and neurotoxicity, and/or enhancing the antitumor effect in tumor models of liver metastases or phagocytic tumors, as has been described already for liposomal doxorubicin (18, 19).

The different liposome compositions tested resulted in changes in drug encapsulation, liposome stability, and antitumor activity. DMPC:DMPG 7:3 proved to be the best lipid composition tested. Compared with DMPC:DMPG 7:3-CPDP, DMPG-CPDP vesicles showed significant structural disruption 14 days after preparation and less antitumor activity against L1210 leukemia, specially when given in a multiple schedule. It appeared that DMPG-CPDP was more toxic than DMPC:DMPG 7:3-CPDP when administered in a multiple schedule to L1210 leukemia-bearing mice. The difference in the in vitro activity between these two preparations was thought to be secondary to the intrinsic in vitro antitumor activity of empty liposomes composed of DMPG. However, empty liposomes composed of DMPG did not have any antitumor activity in vivo.

Further steps in the development of L-CPDP will be directed at optimizing the formulation, assessing the antitumor activity in tumor models in which a targeted delivery of the drug to tumor sites is expected, and studying the modulation of the well-known organ toxicities associated with cisplatin administration.

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