Lipophilic Cisplatin Analogues Entrapped in Liposomes: Role of Intraliposomal Drug Activation in Biological Activity

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ABSTRACT

cis-Bis-neodecanoato-trans-R, R, 1,2-diaminocyclohexane platinum (II) (NDDP), a lipophilic cisplatin analogue containing two branched leaving groups of 10 carbon atoms, is undergoing clinical evaluation in a liposomal formulation. In previous studies, NDDP entrapped in multilamellar vesicles composed of dimyristoylphosphatidylcholine (DMPC) and dimyristoylphosphatidylglycerol (DMPG) at a 7:3 molar ratio was non-nephrotoxic in humans, not cross-resistant with cisplatin in different in vitro and in vivo systems, and more active than cisplatin against murine models of experimental liver metastases whereas free NDDP was devoid of in vivo antitumor activity at the optimal dose of L-NDDP and barely active at higher doses. To elucidate the mechanisms by which the liposomal carrier enhances the biological properties to this class of antitumor agents, we studied the effect of the liposome composition, size of the branched leaving groups of the platinum compound, and pH and composition of the aqueous phase on the entrapment efficiency, drug leakage, drug stability, and in vivo toxicity and antitumor activity of different liposomal formulations of these agents. In experiments using normal saline as aqueous phase, the presence of DMPG in the lipid bilayer resulted in a decreased stability and an increased biological activity of NDDP, whereas NDDP entrapped in liposomes composed of DMPC alone (not containing DMPG) was stable but devoid of antitumor activity. In studies with structurally related analogues with branched leaving groups of 5, 6, 7, and 9 carbon atoms, similar trends were observed. In addition, the number of carbon atoms in the leaving groups was directly and inversely related to the entrapment efficiency and stability of the analogues, respectively, independently of lipid composition; increasing the size of the branched leaving groups resulted in an increased in situ degradation of the platinum compound and enhanced biological activity and potency. These results suggest that this class of platinum compounds exerts its biological activity through the formation of active intermediates in situ within the lipid bilayers and that the activation reaction is highly dependent on the presence of DMPG and the size of the lipophilic leaving group.

INTRODUCTION

NDDP is a lipophilic cisplatin derivative synthesized and developed in a liposomal formulation at our institution (1–4). NDDP belongs to the family of the diaminocyclohexane cisplatin analogues (5–8). This family is noted for its lack of cross-resistance, as a result of the cyclohexane group attached to the two amino groups (6). In addition, NDDP has 2 branched aliphatic leaving groups of 10 carbon atoms which confer an increased lipophilicity and affinity for lipid membranes, thus making the compound optimally suited for liposomal formulation.

NDDP entrapped in liposomes composed of DMPC and DMPG at a 7:3 molar ratio (L-NDDP) was found to be non-nephrotoxic in dogs (3), not cross-resistant with cisplatin in vitro and in vivo in different tumor cell systems (2), and more effective than cisplatin in the treatment of experimental liver metastases (1). In a phase I study in humans, the highest tolerated dose of L-NDDP was 312.5 mg/m². The dose-limiting toxicity was myelosuppression, mainly affecting the white blood cell lineage. Other toxicities were mild and consisted of nausea, vomiting, diarrhea, fever, malaise, and transient elevation of liver enzymes. No nephrotoxicity was observed (4).

NDDP is completely insoluble in water. In preclinical studies, we used a suspension of NDDP in normal saline and 2% Tween 20 to compare the biological activities of free and liposome-entrapped NDDP and to ascertain the role of the liposome carrier in determining the biological activity of this agent. In in vitro studies with human colon carcinoma LoVo cells and their counterparts with acquired resistance to cisplatin, L-NDDP was significantly more cytotoxic than free NDDP against both cell lines (2). In in vivo studies against L1210 and L1210/PDD leukemia (resistant to cisplatin), free NDDP was devoid of antitumor activity at the optimal doses of the liposome-entrapped form and was still significantly less active than the liposomal entrapped form at 3- to 4-fold higher doses (2). These results led to the conclusion that NDDP was a liposome-dependent antitumor agent. We hypothesized that the liposome dependency of NDDP was either due to changes in pharmacokinetics or cellular pharmacology secondary to liposome incorporation or due to an increased or decreased stability of the compound related to a protective or activating effect of the liposomal carrier.

Owing to its high lipophilicity, NDDP is incorporated within the lipid compartment of the lipid vesicles. In an attempt to elucidate the mechanism by which the liposomal carrier enhances the biological activity of NDDP, we explored several variables that affect the liposomal carrier and its interaction with the platinum compound. Specifically, we initially studied the role of the presence of the negatively charged acidic phospholipid DMPG in the lipid bilayer. Subsequently, we investigated the role of the pH and composition of the aqueous phase of the liposome suspension. Finally, we examined the effect of the size of the branched leaving groups by synthesizing and studying compounds with the same general structure as NDDP but with branched leaving groups of 5, 6, 7, and 9 carbon atoms instead of 10. Our results indicate that these compounds are transformed in situ within the lipid bilayers into one or more active intermediates. When normal saline is used as aqueous phase, this degradation/activation step requires the presence of DMPG in the lipid bilayers, is enhanced by increasing the size of the leaving groups, and explains the liposome dependency previously described with this class of platinum compounds.
MATERIALS AND METHODS

Synthesis and Characterization of the Lipophilic Platinum Compounds

NDDP was synthesized as previously reported (9). Fig. 1 shows the chemical structure of NDDP.

Platinum compounds with the same general structure, trans-R,R-1,2-diaminocyclohexane platinum X₂, where X is a branched aliphatic leaving group of 5, 6, 7, or 9 carbon atoms, were synthesized using the same synthetic scheme. Neopentanoic acid, neohexanoic acid, neohexadecanoic acid, and neononanoic acid were purchased from Exxon Corp., Houton, TX. Fig. 2 shows the structure of the leaving groups of these compounds.

All compounds were characterized by elemental analysis, 195Pt nuclear magnetic resonance, and IR-spectroscopy. All compounds were >95% pure, as assessed by high performance liquid chromatography.

Preparation and Characterization of Liposomes Containing Lipophilic Platinum Compounds

DMPC and DMPG were obtained from Avanti Polar Lipids (Pelham, AL). All liposomal formulations were suspensions of multilamellar vesicles. The following lipid compositions were used: DMPC only; DMPC:DMPG at a 7:3 molar ratio; DMPC:DMPG at a 3:7 molar ratio; and DMPG only. All liposomal preparations were obtained by hydration of previously prepared lyophilized mixtures of the lipids and the platinum compounds. The lipid:drug weight ratio was in all cases 15:1. The procedure for preparing the liposomal formulations has been reported previously in detail (3, 10).

To hydrate the lyophilized mixtures, we used either normal saline (0.9% NaCl solution in water, pH 5–6), 5% dextrose in water (pH 5–6), or phosphate buffer (0.05% H₃PO₄ in water, pH 3). The hydration step was always performed by adding 1 ml of aqueous solution per mg of platinum compound to the lyophilized drug-lipid mixture and subsequent mild hand shaking for 1–3 min at room temperature.

All preparations were routinely sized in a Coulter Counter and channelizer (Coulter Electronics, Hialeah, FL) and examined by optical microscopy to rule out the presence of drug aggregates or crystals. The vesicle size ranged from 1 to 5 μm in all cases. Liposome suspensions were examined by optical microscopy to rule out the presence of drug aggregates or crystals. The vesicle size ranged from 1 to 5 μm in all cases. Liposome suspensions were examined by optical microscopy to rule out the presence of drug aggregates or crystals.

The entrapment efficiency was assessed by measuring the amount of elemental platinum in the supernatant after centrifugation of the liposome suspension at different time points after liposome preparation at room temperature. The percentage of drug leakage was calculated as:

\[
\% \text{EE} = \frac{\text{Total initial Pt} - \text{Pt in supernatant}}{\text{Total initial Pt}} \times 100
\]

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\]
Antitumor Activity against L1210 and L1210/PDD Leukemias and M5076 Reticulosarcoma. In vivo antitumor activity studies against L1210 and L1210/PDD leukemias and M5076 reticulosarcoma were performed as previously described. C57BL × DBA/2 F₁ and C57BL/6 mice weighing 18–20 g were purchased from Harlan. L1210 and L1210/PDD cells were obtained from the Tumor Repository, National Cancer Institute, Bethesda, MD. M5076 cells were obtained from the Department of Cell Biology, M. D. Anderson Cancer Center. All three cell lines were kept in vivo as ascitic tumors. L1210 and L1210/PDD leukemias and M5076 reticulosarcoma were regularly transplanted every 3–4 weeks. Groups of 6–8 animals were used as controls. Untreated animals were used as controls. Control groups with animals treated with empty liposomes have no antitumor activity against any of these tumor systems (10). Tumor cells (1 × 10⁶) were inoculated i.p. for the L1210 and L1210/PDD experiments and i.v. (2 × 10⁶) for the M5076 experiments. Treatment consisted of one single i.p. injection on day 1 (L1210); three i.p. injections on days 1, 5, and 9 (L1210/PDD); or three i.v. injections on days 4, 8, and 12 (M5076). Results were expressed as:

\[
\% \text{T/C} = \frac{\text{Median survival of treated animals}}{\text{Median survival of control animals}} \times 100
\]

Table 1 shows the percentage of drug remaining entrapped in the liposomes 0, 2, and 6 h after the preparation of different liposomal formulations of the platinum compounds. Drug leakage was calculated as the difference in percentage of drug entrapped between time 0 and 6 h. Drug leakage was found to vary with the size of the branched leaving groups and the liposome composition. The degree of drug leakage from liposomes composed of DMPC alone was minimal but slightly higher in compounds with small branched leaving groups than in compounds with large branched leaving groups (percentage of drug leakage at 6 h: 11.3, 3.6, 0, 0, and 0 for platinum compounds with leaving groups of 5, 6, 7, 9, and 10 carbon atoms, respectively). The presence of DMPG at a DMPC:DMPG molar ratio of either 7:3 or 3:7 resulted in a slightly increased drug leakage in all cases when compared with liposomes composed of DMPC alone. Drug leakage from liposomes containing DMPG tended to be inversely related to the size of the branched leaving groups (drug leakage from liposomes composed of DMPC:DMPG 7:3 at 6 h: 25.9, 9.7, 11.9, 6.0, and 5.0% for platinum compounds with leaving groups of 5, 6, 7, 9, and 10 carbon atoms, respectively). At 6 h, the percentage of drug still entrapped in the lipid vesicles composed of DMPC:DMPG (7:3) was 37.3, 80.7, 80.9, 93.0 and 94.0% for platinum compounds with leaving groups of 5, 6, 7, 9, and 10 carbon atoms, respectively. For liposomes composed of DMPC:DMPG (3:7) the values of this parameter were 52.5, 72.1, 76.3, and 91.1% for the compounds with leaving groups of 5 and 10 (NDDP) carbon atoms.

The drug leakage over time for the two platinum compounds (with branched leaving groups of 9 and 10 carbon atoms) that had initial drug entrapment values >99% in liposomes of different composition was minimal in all cases: 0% for liposomes composed of DMPC alone; and 5–8% for liposomes containing DMPG.
Stability of Lipophilic Platinum Compounds Entrapped in Liposomes of Different Composition

Fig. 4 shows the stability at 6 h of platinum compounds with branched leaving groups of various sizes entrapped in multilamellar vesicles of different composition. The percentage of drug stability represents the percentage of drug remaining intact in the liposome suspension at different time points after formation of the suspension.

Drug stability was highly dependent on liposome composition and size of the branched leaving groups. The percentage of drug stability was close to 100% for all compounds when liposomes composed of DMPC alone were used. The presence of DMPG resulted in significant degradation of all compounds except for the compound with leaving groups of 5 carbon atoms. In the compounds with branched leaving groups containing 6, 7, and 10 carbon atoms, this effect was especially pronounced when the molar ratio of DMPC:DMPG was 3:7 or when the liposomes consisted of DMPG alone. Degradation also occurred when the molar ratio was 7:3, but to a lesser extent. However, in the case of the compound with leaving groups of 9 carbon atoms, the 7:3 molar ratio of DMPG resulted in the most degradation.

Increasing the number of carbons in the branched leaving group enhanced drug degradation when liposomes containing different amounts of DMPG were used (drug stability at 6 hours was 100, 75–100, 35–60, 50–60, and 0–35% for compounds with leaving groups of 5, 6, 7, 9, and 10 carbon atoms, respectively).

Effect of Lipid Composition and pH and Composition of the Aqueous Phase on Drug Stability, Antitumor Activity, and Subacute Toxicity of NDDP

Table 2 shows the results of drug stability, antitumor activity, and subacute toxicity of different liposomal formulations of NDDP.

NDDP was stable when entrapped in liposomes composed of DMPC alone suspended in 0.9% NaCl solution in water (drug stability at 6 h, 97%). However, this liposomal formulation of NDDP was completely inactive against i.p. L1210 leukemia up to a dose of 50 mg/kg (higher doses were not tested) and the subacute LD50 in ICR Swiss mice was not reached at a dose of 100 mg/kg. The use of phosphate buffer (pH 3) as aqueous milieu resulted in a marked loss of detectable NDDP (drug stability at 6 h, 55%) and significant antitumor activity against i.p. L1210 leukemia at a dose of 25 mg/kg (%T/C 194). A dose of 50 mg/kg was toxic. The degradation rate was highest during the first 2 h after liposome formation.

NDDP entrapped in liposomes containing different proportions of DMPG in 0.9% NaCl solution in water showed significant degradation. Most drug degradation occurred during the first 2 h after liposome formation. Drug degradation was 100% in all DMPG formulations except for DMPC:DMPG (7:3) (drug stability at 6 h, 35%). All liposomal formulations containing DMPG showed significant and similar antitumor activity against i.p. L1210 leukemia (%T/C at optimal dose tested, 233 for DMPC:DMPG (7:3), 193 for DMPC:DMPG (3:7), and 180 for DMPC alone). Drug potency was associated with drug degradation. For DMPC:DMPG (7:3) (drug stability, 36% at 2 h), the optimal dose was 25 mg/kg. A dose of 50 mg/kg was toxic. The subacute LD50 of this formulation was 60.5 mg/kg. By contrast, for DMPC:DMPG (3:7) and DMPG alone (drug stability, 0% at 2 h), the optimal dose was 12.5 mg/kg, a dose of 25 mg/kg being toxic. The subacute LD50 doses for these formulations were 37.5 and 30.2 mg/kg, respectively.

The stability of NDDP entrapped in liposomes composed of DMPC:DMPG (7:3) was similar when two different isotonic aqueous solutions were used: 0.9% NaCl or 5% dextrose (drug stability at 6 h, 35% for 0.9% NaCl solution and 49% for 5% dextrose solution in water). The optimal dose for both preparations was 25 mg/kg. The antitumor activity of both liposome suspensions was also similar (%T/C at optimal dose, 233 for 0.9% NaCl solution versus 200 for 5% dextrose solution).

Table 2 Drug stability, antitumor activity, and toxicity of different liposomal formulations of NDDP

<table>
<thead>
<tr>
<th>Lipid composition</th>
<th>Aqueous solution (pH)</th>
<th>% of stability*</th>
<th>% T/C*</th>
<th>LD50 (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 h</td>
<td>6 h</td>
<td>24 h</td>
<td>12.5 mg/ml</td>
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<tr>
<td>DMPC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.9% NaCl (5–6)</td>
<td>100</td>
<td>97</td>
<td>86</td>
<td>ND*</td>
</tr>
<tr>
<td>0.05% H3PO4 (3)</td>
<td>70</td>
<td>55</td>
<td>45</td>
<td>ND</td>
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<tr>
<td>DMPC:DMPG 7:3</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.9% NaCl (5–6)</td>
<td>36</td>
<td>35</td>
<td>28</td>
<td>ND</td>
</tr>
<tr>
<td>5% dextrose (5–6)</td>
<td>64</td>
<td>49</td>
<td>ND</td>
<td>157</td>
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<tr>
<td>DMPC:DMPG 3:7</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>0.9% NaCl (5–6)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>193</td>
</tr>
<tr>
<td>DMPC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.9% NaCl (5–6)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>180</td>
</tr>
</tbody>
</table>
| * Results are means of 2 independent experiments.
| a L1210 leukemia: tumor inoculation i.p. on day 0. Treatment i.p. on day 1. Results are means of 2 independent experiments.
| c ND, not done.
Table 3 Antitumor activity of different liposomal formulations of platinum compounds against L1210 leukemia

<table>
<thead>
<tr>
<th>No. of carbon atoms in leaving groups</th>
<th>Lipid composition</th>
<th>%T/C a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12.5 mg/kg</td>
<td>25 mg/kg</td>
</tr>
<tr>
<td>5</td>
<td>DMPC</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td>DMPC:DMPG (7:3)</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td>DMPC:DMPG (3:7)</td>
<td>128</td>
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<tr>
<td>6</td>
<td>DMPC</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>DMPC:DMPG (7:3)</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td>DMPC:DMPG (3:7)</td>
<td>156</td>
</tr>
<tr>
<td>7</td>
<td>DMPC</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>DMPC:DMPG (7:3)</td>
<td>173</td>
</tr>
<tr>
<td></td>
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<td>236</td>
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<tr>
<td>9</td>
<td>DMPC</td>
<td>111</td>
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<tr>
<td></td>
<td>DMPC:DMPG (7:3)</td>
<td>142</td>
</tr>
<tr>
<td></td>
<td>DMPC:DMPG (3:7)</td>
<td>171</td>
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<tr>
<td>10</td>
<td>DMPC</td>
<td>100</td>
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<tr>
<td></td>
<td>DMPC:DMPG (7:3)</td>
<td>187</td>
</tr>
<tr>
<td></td>
<td>DMPC:DMPG (3:7)</td>
<td>200</td>
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</table>

a Means of two independent experiments. Cisplatin at the optimal dose of 10 mg/kg results in a %T/C of 162–250.

Effect of Lipid Composition and Size of the Branched Leaving Groups on the Antitumor Activity of Lipophilic Platinum Compounds Entrapped in Liposomes

L1210 Leukemia. Table 3 shows the results of antitumor activity of different liposomal formulations of lipophilic platinum compounds against L1210 leukemia. Animals bearing ascitic L1210 leukemia were treated i.p. on day 1. The doses used were 12.5, 25, and 50 mg/kg. Within the dose range studied, all liposomal formulations composed of DMPC alone were devoid of antitumor activity, except in the case of the compound with a branched leaving group of 5 carbon atoms which showed mild activity (%T/C 142). The antitumor activity of this compound was not significantly influenced by adding DMPG to the liposome composition but was lower than those of the DMPG-containing formulations of compounds with branched leaving groups of 7, 9, and 10 carbon atoms as described below.

The presence of DMPG conferred significant antitumor activity to the compounds with leaving groups of 6, 7, 9, and 10 carbon atoms at the doses studied. A higher relative content of DMPG was associated with a slightly increased antitumor activity for the compound with a 6-carbon leaving group (%T/C for DMPC:DMPG (7:3) and DMPC:DMPG (3:7), 142 versus 171, respectively, at a dose of 50 mg/kg). For compounds with leaving groups of 7, 9, and 10 carbon atoms, a higher relative content of DMPG was associated with a higher potency but not with an increased maximal antitumor activity. For the DMPC:DMPG (7:3) formulations, the optimal doses of the compounds with leaving groups of 7, 9, and 10 carbon atoms were 50, 50, and 25 mg/kg, respectively. The %T/C at these doses were 206, 185, and 200, respectively. For the DMPC:DMPG (3:7) formulations, the optimal doses for the same compounds were 25, 25, and 12.5 mg/kg. The %T/C obtained with these doses was similar to that obtained with the DMPC:DMPG (7:3) formulations: 236, 171, and 200, respectively.

At the doses studied, the compounds which displayed higher antitumor activity were those with leaving groups of 7 and 10 carbon atoms (%T/C 200–236). Results with these compounds were similar to those obtained with cisplatin (%T/C 162–250 in numerous experiments).

L1210/PDD Leukemia. Table 4 shows the antitumor activity of liposomal formulations of platinum compounds with different relative content of DMPG against L1210/PDD leukemia. Treatment in these experiments was administered i.p. on days 1, 5, and 9. Increasing the DMPG content from a DMPC:DMPG ratio of 7:3 to 3:7 was associated with an increased antitumor activity or an increased potency in all cases. For compounds with leaving groups of 5 and 6 carbon atoms, increasing the DMPG content resulted in higher %T/C values at the optimal dose of platinum compound.

For compounds with leaving groups of 7 and 9 carbon atoms, a higher DMPG content resulted in an increased potency of the compounds: the optimal dose for the DMPC:DMPG (7:3) formulations was 25 mg/kg on days 1, 5, and 9; by contrast, for the DMPC:DMPG (3:7) formulations, the optimal dose was 12.5 mg/kg on days 1, 5, and 9; a dose of 25 mg/kg on the same days was toxic.

The highest level of antitumor activity was observed with the compound with leaving groups of 9 carbon atoms entrapped in liposomes composed of DMPC:DMPG (3:7) (%T/C 505). The %T/C with NDDP (10-carbon leaving group) entrapped in liposomes composed of DMPC:DMPG (7:3) was 200. The DMPC:DMPG (3:7) formulation of NDDP was not tested. As expected, cisplatin was devoid of antitumor activity against this tumor model (%T/C 112).

M5076 Reticulosarcoma. Table 5 shows the antitumor activity of different liposomal formulations of the platinum compounds with branched leaving groups of 7 and 10 carbon atoms (NDDP) against M5076 reticulosarcoma. Treatment in these experiments was administered i.v. on days 4, 8, and 12. The doses ranged from 6.25 to 25 mg/kg. Higher doses were not tested.

Table 4 Antitumor activity of different liposomal formulations of platinum compounds against L1210/PDD leukemia

<table>
<thead>
<tr>
<th>No. of carbon atoms in leaving groups</th>
<th>Lipid composition</th>
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</tr>
</thead>
<tbody>
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<td></td>
<td>12.5 mg/kg x 3</td>
<td>25 mg/kg x 3</td>
</tr>
<tr>
<td>5</td>
<td>DMPC:DMPG (7:3)</td>
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</tr>
<tr>
<td></td>
<td>DMPC:DMPG (3:7)</td>
<td>139</td>
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<td>6</td>
<td>DMPC:DMPG (7:3)</td>
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<td>10</td>
<td>DMPC:DMPG (7:3)</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>DMPC:DMPG (3:7)</td>
<td></td>
</tr>
</tbody>
</table>

a Means of two independent experiments. Cisplatin at the optimal dose of 6 mg/kg for (x3) results in a %T/C of 100–115.

Table 5 Antitumor activity of different liposomal formulations of platinum compounds against M5076 reticulosarcoma

<table>
<thead>
<tr>
<th>No. of carbon atoms in leaving groups</th>
<th>Lipid composition</th>
<th>%T/C a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.25 mg/kg x 3</td>
<td>12.5 mg/kg x 3</td>
</tr>
<tr>
<td></td>
<td>25 mg/kg x 3</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>DMPC</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>DMPC:DMPG (7:3)</td>
<td>137</td>
</tr>
<tr>
<td></td>
<td>DMPC:DMPG (3:7)</td>
<td>177</td>
</tr>
<tr>
<td></td>
<td>DMPG</td>
<td>172</td>
</tr>
<tr>
<td>10</td>
<td>DMPC</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>DMPC:DMPG (7:3)</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>DMPC:DMPG (3:7)</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>DMPG</td>
<td></td>
</tr>
</tbody>
</table>

a Means of two independent experiments. Cisplatin at the optimal dose of 6 mg/kg x 3 results in a %T/C of 149–173.
The formulations containing only DMPC were not active at the doses tested. The presence of DMPC conferred significant antitumor activity to the formulations. Increasing the relative content of DMPC resulted in an increased antitumor activity and/or potency of the formulations. In the case of the compound with leaving groups of 7 carbon atoms, the highest %T/C with the DMPC:DMPC (7:3) formulation was obtained at a dose of 25 mg/kg (%T/C 173); with the DMPC:DMPC (3:7) formulation, the dose of 25 mg/kg was toxic and a similar %T/C (177) was observed at a dose of 12.5 mg/kg. In the case of the compound with leaving groups of 10 carbon atoms (NDDP), the DMPC:DMPC (7:3) formulation showed no antitumor activity at 6.25 and 12.5 mg/kg and was toxic at 25 mg/kg; by contrast, the DMPC:DMPC 3:7 formulation was active at both 6.25 and 12.5 mg/kg (%T/C 160 and 191, respectively).

The %T/C obtained with cisplatin ranges between 149 and 173 in numerous experiments.

DISCUSSION

The results obtained strongly suggest that the antitumor activity of liposome-entrapped NDDP is exerted through chemical activation of the platinum compound within the liposomes. When normal saline is used as aqueous solution, the activation reaction is dependent on the presence and relative content of the acidic phospholipid DMPC within the lipid bilayers. In studies with similar compounds with branched leaving groups of different size, the activation rate tended to increase with the size of the branched leaving groups. The compound with a leaving group of 5 carbon atoms was very stable independently of the presence of DMPC and displayed significant antitumor activity. However, its entrapment efficiency was significantly lower than that of the other compounds.

Liposome-entrapped NDDP is an example of a carrier-dependent antitumor agent, or more specifically a liposome-composition-dependent antitumor agent. Previous examples of liposome composition-dependent antitumor agents have been described. In those cases, the liposome composition enhanced the antitumor activity of the agents by altering the pharmacokinetics and organ distribution of the drugs but not by chemical activation of the agent. ara-C encapsulated in liposomes constitutes one such example (11, 12). The presence of cholesterol within the lipid bilayers was found to be essential for ara-C to exert its biological activity when encapsulated in liposomes and administered in a bolus injection. Liposomes containing cholesterol are slowly destroyed in vivo and therefore constitute a much more effective slow drug release system for S-phase-dependent agents (13). However, similar antitumor activity can be observed with free ara-C if the proper administration schedule is used. The “stealth” liposomes constitute a second example of antitumor activity dependent on the liposome composition. The lipid compositions of the “stealth” liposomes prevent the encapsulated drugs from interacting with the serum components and help the liposomes avoid recognition by phagocytes; as a result, these liposomes have shown significant tumor-targeting properties (14).

It is widely accepted that all platinum compounds exert their antitumor activity by reaction of their aminated species with DNA (15). The markedly reduced in vivo antitumor activity and toxicity of free NDDP and NDDP entrapped in liposomes composed of DMPC alone in normal saline may indicate that the aquation reaction of NDDP in vivo is extremely slow when these formulations are used. Free NDDP in suspension in 2% Tween 20 or incorporated in neutral vesicles composed of DMPC alone may partition or be delivered in toto to serum lipoproteins or biological membranes as a result of liposome-cell membrane interchange and not have a chance to interact with water and undergo activation. The constant associations in the different formulations tested of (a) presence of DMPC within the lipid bilayers with NDDP degradation and (b) NDDP degradation with antitumor activity and the very low drug leakage in the presence of significant NDDP degradation strongly indicate that one or several active intermediates of NDDP are formed within the lipid bilayers as a result of a chemical reaction between NDDP and DMPC and that these active intermediates remain liposome bound after formation. Possible active intermediates include diaminocyclohexaneplatinum compounds containing either (a) two chlorides or DMPC molecules as leaving groups, (b) one chloride or DMPC molecule as one leaving group and a neodecanoato group as the other, or (c) the aminated species of NDDP themselves. Compounds containing chloride as leaving groups are likely when normal saline is used as aqueous solution; these compounds are well known to be active. However, because the antitumor activity of L-NDDP reconstituted in 5% dextrose is similar to that obtained when normal saline is used, the formation of chlorinated intermediates is not an essential requirement for NDDP to exert its antitumor activity. Diaminocyclohexaneplatinum-phospholipid complexes have been reported previously as having significant antitumor activity (16). Their formation could, therefore, account for the antitumor activity of L-NDDP.

The observation that the size of the leaving group was an important determinant of the activation reaction, the larger the size of the leaving group the higher the activation, indicates that the size of the leaving group plays an important role in determining the interaction between the platinum compound and DMPC. This is supported by the fact that compounds with smaller leaving groups also had a lower drug entrapment. It would therefore appear that compounds with a large leaving group would be more closely associated with DMPC and this would result in an optimal entrapment and increased chemical reactivity.

Because of its lack of cross-resistance with cisplatin, enhanced transmembrane transport (17), and particular biodistribution [liver, and spleen after i.v. administration, increased retention in peritoneal cavity after i.p. administration (18), etc.], liposome-entrapped NDDP remains a promising new antitumor agent that deserves to be tested in humans for those indications that exploit the particular pharmacological properties of liposomes. The main practical implication of the results of this study is the need for a complete chemical characterization of the liposomal formulation of NDDP in conjunction with its ongoing clinical development. We are currently working on the identification of the common active intermediates of platinum compounds with branched leaving groups. Once identified, they will be synthesized and liposomal formulations prepared and tested for drug stability and antitumor activity. In addition, we are also examining the stability, formulation properties, and antitumor activity of liposomal formulations of platinum compounds with linear leaving groups. All these studies, it is hoped, will clarify in detail the chemical events that need to take place within the lipid vesicles for NDDP to exert its antitumor activity.
REFERENCES


Lipophilic Cisplatin Analogues Entrapped in Liposomes: Role of Intraliposomal Drug Activation in Biological Activity

Roman Perez-Soler and Abdul R. Khokhar


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