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Toxicity and efficacy studies on a series of lipid-soluble dineodecanoato(*trans-R,R-* and *trans-S,S-*1,2-diaminocyclohexane) platinum (II) complexes entrapped in liposomes

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A number of highly lipophilic dineodecanoato(*trans-R,R-* and *trans-S,S-*1,2-diaminocyclohexane) platinum (II) complexes were entrapped in multilamellar vesicles composed of dimyristoyl phosphatidylcholine and dimyristoyl phosphatidyl-glycerol at a molar ratio of 7:3. The entrapment efficiency and stability of liposomal platinum (L-Pt) preparations was >90%. The subacute mouse LD_{50} of L-Pt preparations tested ranged from 60 to 104 mg/kg. All L-Pt preparations tested had no significant nephrotoxicity at the LD_{50} dose except for L-Pt 5, which caused renal dysfunctions (as evidenced by elevated blood urea nitrogen levels) at the LD_{50} dose. L-Pt preparations had shown good *in vivo* antitumor activity against i.p. L1210 leukemia when an optimal dose was administered i.p. to mice on days 1, 5 and 9 (% T/C 230–300; cisplatin 220). L-Pt preparations were also markedly active, by the i.p. route, against L1210 leukemia resistant to cisplatin (% T/C 237–355; cisplatin 112). All L-Pt preparations exhibited significant antitumor activity against B16 melanoma when administered i.p. on day 1 (% T/C 144–155; cisplatin 161). L-Pt 1, 3 and 5 were all tested by the i.v. route on days 4, 8 and 12 against M5076 reticulosarcoma, but none of these preparations showed any significant antitumor activity against this tumor system (% T/C 120–127; cisplatin 173). Current studies aimed at optimizing the liposomal formulation of these compounds should result in the selection of a single isomeric L-Pt formulation for clinical development.

Key words: Antitumor activity, entrapment, liposome, platinum, toxicity.

Introduction

Liposomes are microscopic phospholipid vesicles that naturally target phagocyte-rich organs that have fenestrated capillaries such as liver, spleen and bone marrow.[1–4] Recently, liposomes have been used as drug carriers for certain therapeutic agents since they have the potential to reduce certain drug-related toxicities[4] and enhance anticancer activity.[5,6] Entrapment of drugs in liposomes alters their pharmacokinetics, organ distribution and metabolism. Liposomes may rationally be used for many vehicular purposes; for instance, they can transport poorly soluble drugs, deliver drugs that are less toxic or more active when administered using a slow release system, deliver macrophage activator to macrophage and deliver cytotoxic intracavity agents.

Cisplatin is one of the most useful anticancer drugs[7,8] available, but its clinical usefulness is limited by its development of resistance in marginally sensitive tumors. Its major toxicities are acute nephrotoxicity and chronic neurotoxicity. Therefore, in recent years, there has been a growing interest in developing new platinum complexes that have a broader spectrum of activity and that lack cross-resistance with cisplatin.[9]

1,2-Diaminocyclohexaneplatinum(II), dichloride (dach-$PtCl_2$),[10] a cisplatin analog, has been available for almost two decades. It is non-nephrotoxic and non-cross resistant with cisplatin.[11,12] Unfortunately, it is completely insoluble in water and most organic solvents and, therefore, cannot be formulated for i.v. administration. Its water-soluble derivatives have been previously developed and tested.[13,14] However, these studies were limited or not completed because of formulation problems.

We therefore undertook a drug synthesis program aimed at developing lipophilic cisplatin analogs specifically designed for liposome entrapment with decreased host toxicity and non-cross-resistance with cisplatin. As a result of this ongoing program, we have prepared a liposomal formulation

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of one of these complexes, *cis*-bis(neodecanoato)-(*trans*-R-R-1,2-diaminocyclohexane) platinum (II) (NDDP), which was selected for further development. The preclinical antitumor efficacy of liposomal NDDP (L-NDDP) has been previously reported.[15–17] Briefly, L-NDDP was found to be non-nephrotoxic and more effective than cisplatin against liver and spleen metastases of M5076 reticulosarcoma and active against tumor models resistant to cisplatin.

A clinical phase I study approved by the Food and Drug Administration has recently been completed at the MD Anderson Cancer Center.[18] The maximum tolerated dose of NDDP was 312.5 mg/m^2 and the primary dose-limiting toxicity was myelosuppression. No nephrotoxicity was observed.

Since the neodecanoic acid used for the synthesis of NDDP is a mixture of at least 18 isomers, we decided to explore whether similar complexes having single isomers of neodecanoic acid as leaving groups would have toxicological and pharmacological properties similar to those of NDDP. We have previously reported the synthesis, characterization and partial antitumor data against L1210 leukemia on several *trans*-R,R- and *trans*-S,S-DACH-Pt(II) complexes containing a single isomer of neodecanoic acid.[19] We report here on the development of liposomal formulation, the toxicity and the antitumor efficacy against various tumor systems of such platinum complexes. These studies further confirm our previous conclusion that the different isomers of NDDP entrapped in liposomes display significant differences in toxicity and antitumor activity when compared among themselves and also when compared with the racemic mixture of L-NDDP. Extensive liposome formulation studies are now in progress in order to learn how to optimize the biological activity of these compounds.

Materials and methods

Source of platinum complexes

All platinum complexes have previously been synthesized, and their physical, chemical and spectroscopic properties reported;[19] (*trans*-R,R-dach)(dimethyloctanoato)$_2$ platinum (II), **1**; (*trans*-S,S-dach)(dimethyloctanoato)$_2$ platinum (II), **2**; (*trans*-R,R-dach)(2,2-diethylhexanoato)$_2$ platinum (II), **3**; (*trans*-S,S-dach)(2,2-diethylhexanoato)$_2$ platinum (II), **4**; (*trans*-R,R-dach)(2,2-diethyl-4-methylpentanoato)$_2$ platinum (II); **5**; (*trans*-S,S-dach)(2,2-diethyl-4-methylpentanoato)$_2$ platinum (II), **6**. The chemical structures of these platinum complexes are shown in Figure 1.

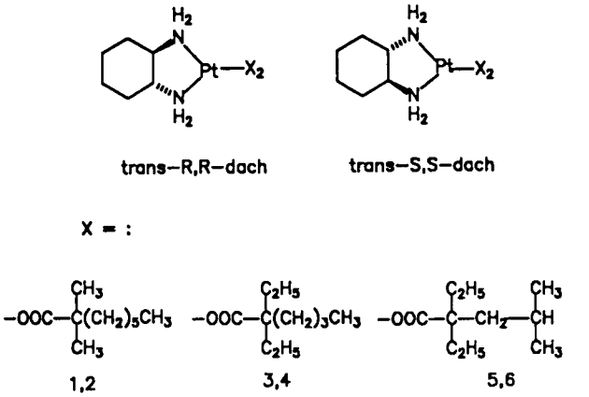

Figure 1. Structure of platinum complexes **1–6**.

Lipids

The chromatographically pure [by thin layer chromatography (TLC)] dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG) were obtained from Avanti Polar Lipids (Birmingham, AL).

Animals

CD1 Swiss mice, 6–8 weeks old and weighing 22–25 g each, were purchased from The University of Texas MD Anderson Cancer Center, Science Park (Smithsville, TX). BDF1 and C57BL16 mice weighing 18–20 g each were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, MA).

Preparation of liposomal platinum

Liposomal-platinum (L-Pt) formulations were prepared as lyophilized powders containing DMPC, DMPG and the different platinum complexes (10.5:4.5:1 weight ratio). To obtain the lyophilized powders, lipids and platinum complexes were dissolved in t-butyl alcohol at the above mentioned ratio, and the obtained solutions were frozen and lyophilized overnight. The resulting white, flaky powder was stored at 4°C until use; lyophilized L-Pt preparations were stable at 4°C for at least 4

weeks as assessed by TLC. To obtain liposome suspensions containing platinum complexes, the lyophilized preparations were reconstituted with 0.9% NaCl solution in water (final concentration, 1 mg platinum complex/ml) by mild hand-shaking the mixture at room temperature for 2–3 min.

Toxicity studies

Groups of six to eight CD1 Swiss mice weighing 22–25 g each received different i.v. doses of L-Pt complex. Animals were observed and their deaths recorded on a daily basis. The LD_{50} was calculated from the curve obtained by plotting the logarithm of the dose and the percentage of survival on day 15. Acute nephrotoxicity was evaluated after a single i.v. injection of the calculation LD_{50} dose of each L-Pt complex to CD1 mice by measuring BUN values at 4 days.

Efficacy studies

To assess the *in vivo* antitumor activity of L-Pt complexes, groups of six BDF1 mice were inoculated i.p. with 10^6 L1210/0 cells or 10^6 L1210/cisplatin cells, and treatment was initiated 24 h later (day 1). Each L-Pt complex was administered in three i.p. injections on days 1, 5 and 9, respectively. Antitumor efficacy of each agent was expressed as % T/C, as determined by the following formula:

$$\%\,T/C = \frac{\text{median survival time of the treated mice}}{\text{median survival time of the control mice}} \times 100$$

The antitumor activity of the L-Pt complexes was also measured against i.p. inoculated B16 melanoma cells. Suspensions of B16 melanoma cells were obtained by homogenizing s.c. inoculated B16 melanoma tumors with phosphate-buffered solution (approximately 10 ml/g tumor) at 4°C. C57BL/6 mice were inoculated i.p. with 0.1 ml of the B16 melanoma cell suspension on day 0. Treatment was given i.p. on day 1. The results were expressed as % T/C, as shown above.

To assess the *in vivo* oncolytic activity of L-Pt complexes against M5076 reticulosarcoma, groups of six to eight C57BL/6 mice were inoculated i.v. via tail vein on day 0 with 2×10^4 M5076 cells obtained from the peritoneal cavity of tumor-bearing animals. L-Pt complexes were injected i.v. on days 4, 8 and 12 in volumes ranging from 0.2 to 0.5 ml. Results were expressed as % T/C.

Results

Entrapment efficiency and stability of L-Pt complexes

The entrapment efficiency of L-Pt complexes measured by UV spectrophotometry[16] was >90%. In addition, no precipitation of free drug could be seen by optic microscopy. The stabilty of L-Pt complexes in normal saline solution at 4°C was assessed by measuring the amount of free platinum complex in the supernatant obtained after centrifugation at 20 000 ***g*** for 45 min at 4°C. Free platinum complexes were measured by UV spectrophotometry at 224 nm. On day 14, the stability was found to be >90% in each case and no evidence of vesicle disruption or clumping of non-entrapped drug was observed by light microscopy. Vesicles ranged in size from 0.5 to 5 μm, with most vesicles measuring between 1 and 3 μm.

Toxicity studies

Two types of toxicity studies were performed with each drug. Initially, CD1 mice were administered single i.v. injections of different doses of the various L-Pt complexes and survival was monitored. The LD_{50} value was calculated from the curve obtained by plotting the logarithm of the dose and the percentage of survival on day 15 (Table 1). All L-Pt complexes proved significantly less toxic than cisplatin (L-Pt complexes, 62–104 mg/kg; cisplatin,

Table 1. Toxicity studies with L-Pt complexes

L-Pt complexes	LD_{50} (mg/kg)	BUN[a] (mg/100 ml)
1	94.5	26.3 ± 4.2
2	87.0	36.4 ± 6.2
3	80.8	28.5 ± 5.7
4	104.0	23.0 ± 4.3
5	62.3	45.6 ± 8.9
6	81.0	30.0 ± 3.6
L-NDDP	56.0	30.5 ± 2.2
Cisplatin	17.5	78.3 ± 8.0

[a] Measured 4 days after single i.v. injection of the LD_{50} dm; normal value 31 ± 5.5 mg/100 ml.

17 mg/kg). The acute nephrotoxicity was then evaluated by administering the calculated LD_{50} dose of each L-Pt complex to CD1 mice in a single i.v. injection and BUN values were determined 96 h later. As shown in Table 1, only mice that were administered cisplatin or L-Pt (**5**) had BUN levels significantly greater than controls.

Efficacy studies

In vivo antitumor activity studies were initially performed against i.p. inoculated L1210 leukemia cells. L-Pt complexes administered i.p. on days 1, 5 and 9 at the optimal doses (25 mg/kg/day) resulted in % T/C values varying between 230 and 300, while the % T/C with cisplatin was 220 (Table 2).

The efficacy of the L-Pt complexes was subsequently evaluated against i.p inoculated L1210/cisplatin leukemia cells (Table 3). When administered i.p. on days 1, 5 and 9 after tumor inoculation, all six L-Pt complexes showed significant antitumor activity, whereas cisplatin was inactive (% T/C 185–355 versus 112).

Table 2. *In vivo* efficacy of L-Pt complexes against L1210 leukemia[a]

L-Pt complex	Treatment schedule days	Optimal dose (mg/kg)	% T/C
1	1, 5, 9	25	257
2	1, 5, 9	25	287
3	1, 5, 9	25	257
4	1, 5, 9	25	230
5	1, 5, 9	25	257
6	1, 5, 9	25	300
Cisplantin	1, 5, 9	5	220

[a] i.p. tumor inoculation on day 0, i.p. treatment.

Table 3. Efficacy of L-Pt complexes against L1210/cisplatin leukemia[a]

L-Pt complex	Treatment schedule days	Optimal dose (mg/kg)	% T/C
1	1, 5, 9	25	355
2	1, 5, 9	25	338
3	1, 5, 9	25	231
4	1, 5, 9	25	237
5	1, 5, 9	25	185
6	1, 5, 9	25	250
Cisplantin	1, 5, 9	5	112

[a] i.p. tumor inoculation on day 0, i.p. treatment.

Table 4. Antitumor activity of L-Pt complexes against B16 melanoma[a]

L-Pt complex	Treatment schedule day	Optimal dose (mg/kg)	% T/C
1	1	25	144
2	1	25	150
3	1	25	150
4	1	25	155
5	1	25	155
6	1	25	155
Cisplantin	1	10	161

[a] Mice were inoculated i.p. with 0.2 ml of B16 melanoma cells (day 0), and the L-Pt were administered i.p. on day 1.

The antitumor activity was also assessed against i.p. inoculated B16 melanoma cells. Table 4 shows the results obtained after single dose i.p. administration of the L-Pt complexes. The antitumor activity of these complexes was similar to that of cisplatin (% T/C 144–155 versus 161).

Table 5 shows the results of antitumor activity against M5076 reticulosarcoma at the optimal dose of selected L-Pt complexes. In this model, tumor cells were inoculated i.v. on day 0, and treatment was given on days 4, 8 and 12. The three L-Pt complexes tested were inactive against this tumor system (% T/C 125–127 vs. 173).

Discussion

We have previously reported extensively on different chemical, biological, toxicological and clinical studies with L-NDDP.[16,17] These studies have provided a pharmacological rationale for the use of this agent for the treatment of tumors that involve the organs of the reticulo-endothelial system, intracavitary tumors and tumors that are resistant to cisplatin, and have shown that the

Table 5. Treatment of experimental liver metastases of M5076 reticulosarcoma[a]

L-Pt complex	Treatment schedule days	Optimal dose (mg/kg)	% T/C
1	4, 8, 12	6.25	120
3	4, 8, 12	12.5	122
5	4, 8, 12	12.5	127
Cisplantin	4, 8, 12	6	173

[a] C57BL/6 mice were inoculated i.v. with 2×10^4 M5076 cells on day 0. L-Pt treatment was administered i.v.

administration of L-NDDP to humans is safe. However, NDDP is a mixture of about 20 different platinum species having different isomers of neodecanoic acid as the leaving group. This constitutes an undeniable source of complexity for the chemical characterization of this compound that may prevent adequate documentation of reproducibility among batches and eventually may hinder the widespread clinical development of this agent.

We, therefore, believe that this limitation needs to be addressed in addition to the clinical development efforts currently ongoing with this agent. Several single isomers of NDDP were synthesized to study their formulation in liposomes as well as their biological activity.[19] These studies were undertaken to explore the possibility of selecting a single isomer with which to continue the clinical development already started with L-NDDP.[18] Our results show that the different single isomeric L-Pt preparations display significant differences in biological activity. In terms of toxicity, the LD_{50} of the single isomeric L-Pt preparations ranged between 62.3 and 104.0 mg/kg, compared with a previously reported LD_{50} for L-NDDP of 54.0 mg/kg. The different isomeric L-Pt preparations did not result in detectable elevations of the BUN at the LD_{50} dose except in the case of L-Pt **5**. Still, L-Pt **5** was significantly less nephrotoxic than cisplatin. We have previously reported that L-NDDP does not cause BUN elevation at the LD_{50} dose.

Antitumor activity studies also showed some interesting differences among the isomeric L-Pt preparations when tested against L1210/cisplatin leukemia by i.p. administration. Preparations L-Pt **1** and **2** were significantly more active than preparations L-Pt **3–6**. The % T/C values for L-Pt **1** and **2** are significantly higher than those obtained previously for L-NDDP[16] (355, 338 and 200, respectively). The antitumor activity of preparations L-Pt **3–6** and L-NDDP appear to be similar in this tumor model. Against L1210 leukemia and B16 melanoma, the antitumor activity displayed by the different single-isomer L-Pt preparations was similar and comparable to the activity observed previously with L-NDDP.

By contrast, against M5076 reticulosarcoma, none of the single isomeric L-Pt preparations showed significant antitumor activity. We have previously reported that L-NDDP has significant antitumor activity against this model.[16] The reason for this discrepancy is being investigated and preliminary evidence suggests that all the platinum complexes that we have developed for liposome entrapment, including NDDP, need to be activated within the lipid bilayers of the multilamellar vesicles in order to exert their biological activity. The activation process appears to be dependent on the lipid composition used, the pH and Cl concentration of the final liposome suspension, and the spatial structure of the leaving group in the platinum complex. Since the isomeric mixture NDDP and the single isomers studied here differ in some of these characteristics, it is likely that the differential antitumor activity observed after i.v. administration between the isomeric mixture and the single isomers may be related to different degrees of *in situ* activation of the complexes once they are entrapped within the lipid bilayers. Studies to optimize the liposomal formulations of the single isomer platinum complexes presented here are now in progress. These studies should result in formulations with enhanced antitumor activity after i.v. administration and should eventually lead to the selection of a single-isomer L-Pt formulation to substitute for L-NDDP in future clinical development efforts.

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