Cancer Chemother Pharmacol (1996) 37: 435–444

ORIGINAL ARTICLE

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In vivo antitumor activity of *cis*-bis-neodecanoato-*trans*-R,R-1,2-diaminocyclohexane platinum(II) formulated in long-circulating liposomes

Received: 13 November 1994/Accepted: 14 May 1995

Abstract A lipophilic cisplatin derivative, *cis*-bis-neodecanoato-*trans*-*R*,*R*-1,2-diaminocyclohexane platinum(II) (NDDP), was formulated in liposomes composed of phosphatidylcholine (PC) and cholesterol (Chol) additionally containing monosialoganglioside (G_{M1}) or polyethyleneglycol conjugated to phosphatidylethanolamine (PEG-PE). These NDDP-containing long-circulating liposomes were examined for in vivo antitumor activity using the mouse RIF-1 solid tumor as a target residing outside the reticuloendothelial system (RES). Biodistribution studies, using C3H/HeJ mice and ^{111}In-labelled DTPA-SA as a lipid marker, showed that the activity of G_{M1} and PEG-PE in prolonging the circulation times of liposomes was preserved in the presence of 3.0 mol% of NDDP in the liposome membranes. The high levels of liposomes remaining in the blood for PC/Chol/G_{M1} and PC/Chol/PEG3000-PE liposomes were associated with high levels of platinum in the blood as determined by atomic absorption spectrophotometry. These NDDP-containing long-circulating liposomes showed approximately a three-fold increase in tumor accumulation as compared to the conventional PC/Chol liposomes. In vitro cytotoxicity studies using RIF-1 tumor cells showed that the presence of PEG-PE, but not G_{M1}, significantly enhanced the cytotoxicity of liposomal NDDP. RIF-1 tumor-bearing C3H/HeJ mice were treated twice with 25 mg/kg NDDP in various liposomal formulations on days 12 and 16 after tumor cell inoculation. A significant reduction in the tumor growth rate was observed when NDDP was formulated in PC/Chol/PEG3000-PE liposomes which support both efficient tumor accumulation and enhanced cytotoxicity of liposomal NDDP. On the other hand, NDDP formulated in PC/Chol/G_{M1} liposomes, which display only a high tumor accumulation, had no effect on the tumor growth rate. Furthermore, NDDP formulated in dimyristoylphosphatidylglycerol (DMPG)-containing liposomes, exhibiting in vitro cytotoxicity comparable to NDDP formulated in PC/Chol/PEG3000-PE liposomes, but showing poor tumor accumulation, was also not effective. These results indicate a potential effectiveness of NDDP formulated in PEG-PE-containing liposomes for therapy of tumors in non-RES organs.

Key words Drug delivery · Lipophilic cisplatin · Long-circulating liposomes

Abbreviations *Chol* cholesterol · *DMPC* dimyristoyl phosphatidylcholine · *DMPG* dimyristoyl phosphatidylglycerol · *DOPE* dioleoyl phosphatidylethanolamine · *DTPA-SA* diethylenetriamine pentaacetic acid distearylamide complex · *FAAS* flameless atomic absorption spectroscopy · *FCS* fetal calf serum · G_{M1} monosialoganglioside · *HBSS* Hank's balanced salt solution · IC_{50} 50% inhibitory concentration · *NDDP* *cis*-bis-neodecanoato-*trans*-*R*,*R*-1,2-diaminocyclohexane platinum(II) · *PBS* phosphate-buffered saline · *PC* phosphatidylcholine · *PE* phosphatidylethanolamine · *PEG* polyethylene glycol · *PEG-PE* dioleoyl *N*-(monomethoxy polyethyleneglycol succinyl) phosphatidylethanolamine · *RES* reticuloendothelial system

This work was supported by NIH Grants CA 24553 (L.H.), CA 41581 and CA 58342 (R. P.-S.). This work was presented in part at the 85th Annual Meeting of the American Association for Cancer Research, San Francisco, Calif., 10–13 April 1994

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Introduction

The development of liposomes with a reduced affinity for cells in the reticuloendothelial system (RES) and thus with prolonged circulation times in the blood has been a major breakthrough in the field of liposome targeting (for reviews, see references 1–3). This has led to the possibility of targeting liposomes to cells, tissues, or organs other than the RES. Such passive targeting of liposomes to non-RES organs is difficult with conventional liposomes due to their rapid clearance from the circulation (for review, see reference 4). Since the original demonstration by Gabizon and Papahadjopoulos [5] that long-circulating liposomes are able to accumulate efficiently in subcutaneously implanted solid tumors, many studies have explored the potential applications of these liposomes for delivering therapeutic agents to non-RES organs. Particularly, certain solid tumors, containing a leaky vascular structure and thus exhibiting an increased microvascular permeability to macromolecules [6, 7], are the obvious target for long-circulating liposomes containing antitumor drugs [8–11]. In fact, several studies using animal tumor models have shown a superior antitumor effect with decreased toxicity of antitumor drugs by formulating them in long-circulating liposomes [12, 13].

Long-circulating liposomes are constructed by inclusion of a specific amphiphile, such as monosialoganglioside (G_{M1}) [14–16] and amphipathic polyethyleneglycol (PEG) [17–23], in a lipid composition mainly composed of a matrix phospholipid, such as phosphatidylcholine (PC), and cholesterol (Chol). We have previously optimized, using PC/Chol-based liposomes, several variables in the liposome construction for prolonged circulation times [17, 20, 22, 24]. Studies with various PEGs with different chain lengths conjugated to phosphatidylethanolamine (PE) have shown that the activity of PEG-PE in prolonging the circulation times of liposomes is directly proportional to the polymer chain length of PEG [22]. This finding suggests that the mode of action of amphipathic PEG is to effectively reduce interactions of liposomes with serum opsonin proteins, thus resulting in their reduced interactions with the RES organs. On the other hand, the mechanism of G_{M1} in increasing the circulation times of liposomes is not clearly understood, although several hypotheses have been proposed [25–27]. The liposome size has also been shown to play an important role in determining the liposome biodistribution [24, 28–30]. The optimal size appears to be 80–200 nm in diameter at which total accumulation of liposomes in the spleen and liver is minimized [24]. Thus, appropriate selections of both the lipid composition and liposome size are used to obtain the maximal circulation times of liposomes. Our previous studies have shown that several lipophilic antitumor prodrugs can be successfully formulated in these long-circulating liposomes [31].

A lipophilic cisplatin derivative, *cis*-bis-neodecanoato-*trans*-*R*,*R*-1,2-diaminocyclohexane platium(II) (NDDP), has been developed specifically for liposomal formulation [32–38]. The antitumor activity of NDDP formulated in liposomes composed of dimyristoyl phosphatidylcholine (DMPC) and dimyristoyl phosphatidylglycerol (DMPG) has been extensively studied in various tumor models [32–34] and in recent clinical trials [35]. These studies have revealed several characteristics of this liposomal NDDP formulation, such as a lack of nephrotoxicity [34] and of cross-resistance with cisplatin [33]. Importantly, the antitumor activity of liposomal NDDP depends on the presence of DMPG in the liposome membranes [36–38]. It has been suggested that DMPG facilitates the chemical activation of NDDP and thus enhances the antitumor activity of liposomal NDDP [36], although detailed mechanisms have yet to be determined.

The above studies intended to target liposomal NDDP to the liver by taking advantage of the inherent homing activity of multilamellar DMPC/DMPG liposomes to this organ. In the present study, in an attempt to develop liposomal NDDP formulations suitable for targeting to non-RES organs, we formulated NDDP in PC/Chol-based unilamellar liposomes containing either G_{M1} or PEG-PE and tested their in vivo antitumor activity in the mouse RIF-1 solid tumor model [39, 40]. These long-circulating liposomes are expected to deliver increasing amounts of NDDP in the tumor and provide an efficient drug delivery system for tumors residing outside the RES organ.

Materials and methods

Materials

Egg PC and dioleoyl phosphatidylethanolamine (DOPE) were obtained from Avanti Polar Lipids (Birmingham, Ala.). Chol, PEG (average molecular weight 8000) and dextran (average molecular weight 515,000) were from Sigma Chemical Co. (St. Louis, Mo.). Bovine G_{M1}, DMPC and DMPG were obtained from Matreya (Mount Pleasant, Pa), and PEG (molecular weight 1000 and 3000) was from Nippon Oil & Fats Co (Tsukuba, Japan). All other chemicals were of reagent grade. NDDP was synthesized as previously described [41]. Synthesis of dioleoyl *N*-(monomethoxy PEG succinyl) PE (PEG-PE) [20] and diethylenetriamine pentaacetic acid distearylamide complex (DTPA-SA) [42], has been described previously. Radiolabelling of DTPA-SA with ^{111}In was performed as described previously [20].

Cell culture, animal and solid tumor model

The RIF-1 fibrosarcoma model was maintained in vivo by s.c. implantation of tumor tissues into the left hind legs of 6–10-week-old female C3H/HeJ mice (Charles River Laboratories, Wilmington, Mass.) as described previously [40]. The mice were housed in

accordance with institutional guidelines. A single-cell suspension was prepared from tumor tissue dispersed by collagenase and was grown as monolayer cultures in Waymouth's medium supplemented with 15% (v/v) fetal calf serum (FCS), penicillin (200 units/ml) and streptomycin (100 μg/ml). Cultures were maintained at 37° C in a humidified atmosphere of air containing 5% CO_2. Cells were detached from culture flask plates by incubating in 0.05% trypsin/0.53 m*M* EDTA in Hank's balanced salt solution (HBSS) at 37° C. For preparation of tumor-bearing mice, trypsinized RIF-1 tumor cells were washed three times with serum-containing medium and resuspended in Ca^{2+}- and Mg^{2+}-free HBSS at a concentration of 1×10^7 cells/ml. Cell viability was ascertained by trypan blue exclusion. Cells were inoculated s.c. into mice (1×10^6 cells in 100 μl per mouse) using a tuberculin syringe with a 27-gauge 1/2-inch needle. Studies were initiated 10 days after tumor cell inoculation at which time tumors weighed approximately 0.5 g.

Liposome preparation

Large unilamellar liposomes with or without NDDP at 3.0 mol% of the lipid mixture were prepared by an extrusion method. The following lipid compositions were used in this study: PC/Chol (10:5, mol/mol), PC/Chol/G_{M1} (10:5:1, mol/mol), PC/Chol/PEG-PE (10:5:1, mol/mol), PC/Chol/DPPG (10:5:1, mol/mol), and DMPC/DMPG (7:3, mol/mol). The solvent-free lipid mixture containing ^{111}In-labelled DTPA-SA, as a nonexchangeable and non-metabolizable lipid marker [42], at 1.0 mol% of the lipid mixture was hydrated with phosphate-buffered saline (PBS; 1370 m*M* NaCl, 27 m*M* KCl, 15 m*M* KH_2PO_4, 1 m*M* Na_2HPO_4; pH 7.4) overnight. Normally, the liposome suspension (2 mg lipid per ml PBS) was extruded at room temperature about 20 times through stacked Nucleopore membranes (0.4 and 0.2 μm pore size) using LiposoFast (Avestin, Ottawa, Canada) [43] to generate liposomes with a homogeneous size distributions. Liposome size was determined by dynamic laser light scattering using a Coulter N4SD instrument (Hialeah, Fl.) and is expressed as average diameter with SD. The incorporation of NDDP into liposomes with any lipid composition did not cause aggregation or precipitation of drugs and/or liposomes before or after the extrusion procedure, indicating complete incorporation of the drug in all liposomal formulations. All liposomal formulations were used for studies within 6 h of preparation.

Liposome partitioning assay

The assay utilized an aqueous two-phase system composed of PEG and dextran [19, 44]. Briefly, both PEG8000 and dextran were dissolved at 5% (w/w) in 0.01*M* Na-phosphate buffer (pH 6.8) containing 0.15 *M* NaCl ('non-charged' phase system), and the phase system was allowed to equilibrate overnight at room temperature. The PEG-rich upper and dextran-rich lower phases were then separated and kept at 4° C. For the liposome partitioning assay, ^{111}In-labelled liposomes (0.1 mg lipid in 50 μl PBS, pH 7.5) were mixed with equal volumes (1 ml) of the upper and lower phases in a 50 × 10 mm tube. Immediately after mixing by repeated inversion for 1 min, 50 μl of the mixture was sampled for total radioactivity counting. The mixture was left at room temperature for 30 min to allow for phase separation, and 100 μl of each phase was sampled to determine liposome partitioning. The amount of liposomes localized at the horizontal interface was obtained by subtracting the sum of the radioactivity in the upper and lower phases from the total radioactivity. Data are expressed as the percentage of the total liposomes in the upper and lower phases and the interface.

In vitro cytotoxicity assay

Liposomes containing NDDP were prepared as described above. RIF-1 tumor cells were prepared as described above, plated onto 96-well plates at 5×10^3 cells in 100 μl of serum-containing medium per well, and incubated for 24 h at 37° C. After washing the cells, 100 μl of the medium containing various concentrations (0.001–100 μ*M*) of NDDP solubilized in 2% Tween 20 or incorporated into liposomes was added. After incubation for specified periods at 37° C, the cells were washed three times with HBSS, fixed with methanol/acetic acid (3:1, vol/vol), and incubated with crystal violet (Sigma) dissolved at 0.5 mg/ml of 20% ethanol for 15 min. Cells were then extensively washed with PBS (pH 7.4), and lysed by the addition of 100 μl 20% methanol/10% acetic acid. The amount of crystal violet absorbed in the plate was quantitated by measurement of the absorbance at 540 nm using a multiscan auto platereader. Data were analyzed as the percentage of viable cells relative to the control cultures incubated in the medium without drugs. The IC_{50} value, representing the concentration of the drug required to inhibit cell growth by 50%, for each NDDP formulation was determined graphically.

Liposome biodistribution study

^{111}In-labelled liposomes with various lipid compositions were injected i.v. into normal or RIF-1 tumor-bearing C3H/HeJ mice (6–10 weeks old) at a dose of 0.2–0.4 mg lipid per mouse in 0.2 ml PBS (pH 7.5). At specified time intervals, mice were anesthetized, bled by retroorbital puncture, and then sacrificed by cervical dislocation and dissected. Blood, tumor, and major organs including the spleen, liver, lung, heart and kidney were collected and weighed. The biodistribution of the liposomes was determined by analyses of ^{111}In radioactivity in each organ using a Beckman gamma-counter. For analysis of platinum, weighed samples of blood and solid tissues were transferred to polypropylene tubes, solubilized with benzethonium hydroxide at 55° C according to a method described previously [45], and analyzed by flameless atomic absorption spectroscopy (FAAS). Platinum levels in the samples were quantitated using a standard method as described elsewhere [46]. Data are expressed as the percentage of the total injected dose of liposomes in each organ. Liposome levels in the blood were determined by assuming that the blood volume in milliliters of a mouse is 7.3% of the total body weight in grams [47].

In vivo antitumor activity of NDDP incorporated into liposomes

Liposomes containing NDDP were prepared as described above and diluted to 2.5 mg NDDP/ml in PBS. The effect of NDDP incorporated into liposomes on the RIF-1 tumor growth rate was studied by i.v. administration of various NDDP formulations at 25 mg NDDP/kg body weight per treatment. Treatments were given on days 12 and 17 after tumor cell inoculation. Treatment groups of mice (five mice per group) included PBS, NDDP solubilized in 2% Tween 20, or NDDP incorporated into liposomes with various lipid compositions. Treated mice were monitored by measurement of tumor diameters with calipers on days 1, 3, 5, 8, and 12 after the initial treatment. The tumor volume was calculated using the formula: (volume) = $[(\text{length}) \times (\text{width})^2]/2$, and is expressed as a percentage relative to the initial tumor volume.

Statistical analyses

The paired Student's *t*-test was used to assess the level of significance between experimental groups.

Results

Effect of NDDP incorporation on surface polarity of liposomes

Due to the highly hydrophobic properties of NDDP, most of the drug incorporated into liposomes is likely to associate tightly with the liposome membrane. It is thus possible that NDDP incorporation results in altered surface characteristics of liposomes. Since the surface polarity of liposomes has been shown to play an important role in determining biodistribution of liposomes [19, 48], we first sought to determine whether NDDP incorporation affects the surface polarity of liposomes using an aqueous two-phase partitioning assay. The assay utilized the PEG/dextran-based aqueous two-phase system with a selected condition, allowing the relative surface hydrophobicity of the liposomes to be assessed (for review, see reference 49). Three PC/Chol-based liposome formulations, PC/Chol, PC/Chol/G_{M1}, and PC/Chol/PEG3000-PE, with or without NDDP at 3.0 mol% in the lipid mixture were prepared by the extrusion method with average diameters ranging from 148 to 226 nm. Data are expressed as the percentage of total liposomes in the two phases and at the horizontal interface, together with a respective upper/lower ratio (Table 1). PC/Chol and PC/Chol/G_{M1} liposomes were found to localize primarily at the interface and secondarily in the lower phase with upper/lower ratios of 0.02 and 0.05, respectively. On the other hand, PC/Chol/PEG3000-PE liposomes showed enhanced partitioning into the upper phase with an upper/lower ratio of 21, indicating an enhanced hydrophilicity of PEG-PE-containing liposomes. Incorporation of NDDP into liposomes did not alter the partitioning of each liposome formulation, showing a similar upper/lower ratio to the corresponding NDDP-free liposomes. These results indicate that NDDP has little effect on the surface hydrophobicity of liposomes.

Biodistribution of NDDP-containing liposomes

To examine the effect of NDDP incorporation on liposome biodistribution, various PC/Chol-based liposomes with or without NDDP at 3.0 mol% in the lipid mixture were prepared with average diameters ranging from 108 to 212 nm. ^{111}In-labelled liposomes were injected i.v. into mice, and the biodistribution was examined 3 h later. The results are expressed as the percentage of injected dose of liposomes in the blood, spleen and liver, together with a respective RES (spleen + liver)/blood ratio (Table 2). Conventional PC/Chol liposomes were cleared rapidly from the circulation and accumulated efficiently in the spleen and liver with a RES/blood ratio of 4.3. PC/Chol/G_{M1} and PC/Chol/PEG3000-PE liposomes showed higher levels of liposomes in the blood with a concomitant decrease in RES accumulation as compared to PC/Chol liposomes. The activities of G_{M1} and PEG3000-PE in prolonging the circulation time of liposomes appeared to be comparable, as indicated by their similar RES/blood ratios (0.5 and 0.4, respectively). The activity of PEG1000-PE in prolonging the circulation time of liposomes was lower than that of PEG3000-PE, in agreement with the previous observation that the activity of PEG-PE in prolonging the circulation times of liposomes is directly proportional to the PEG chain length [31]. On the other hand, two DMPG-containing liposome formulations, PC/Chol/DMPG and DMPC/DMPG, showed even more rapid clearance than PC/Chol liposomes. Incorporation of NDDP into liposomes at 3.0 mol% in the lipid mixture did not cause a significant alteration in their biodistribution irrespective of the lipid composition. Thus, biodistribution of NDDP-containing liposomes is determined essentially by the parent lipid composition.

Disposition of platinum

Because of the high affinity of NDDP to liposome membranes, it was expected that the disposition profile

Table 1 Partitioning of liposomes containing NDDP in the aqueous two-phase system comprising 5% (w/w) PEG/5% (w/w) dextran in 0.01 *M* Na-phosphate buffer (pH 6.8) containing 0.15 *M* NaCl. The composition of the liposome preparations in terms of lipid molar ratios are described in Materials and methods. Values are means (SD), $n = 3$

Lipid composition	% liposome added			
	Upper phase	Interface	Lower phase	Upper/lower
PC/Chol	0.5 (0.4)	71.8 (4.4)	27.7 (4.1)	0.02
PC/Chol + NDDP	0.6 (0.1)	69.6 (3.3)	29.8 (3.4)	0.02
PC/Chol/G_{M1}	0.9 (0.8)	81.5 (3.6)	17.6 (4.1)	0.05
PC/Chol/G_{M1} + NDDP	0.9 (0.1)	82.1 (7.6)	17.0 (7.6)	0.05
PC/Chol/PEG3000-PE	53.3 (4.7)	43.8 (4.2)	2.6 (0.9)	20.5
PC/Chol/PEG3000-PE + NDDP	49.9 (3.6)	47.9 (3.9)	2.2 (0.3)	22.7

of NDDP would depend on the biodistribution of liposomes. It is also possible that liposomal NDDP undergoes metabolic conversions at sites of drug accumulation, followed by redistribution of the metabolites to other organs. To examine the tissue disposition of NDDP and its metabolites, ^{111}In-labelled liposomes containing NDDP were injected into mice, and the platinum level in major organs was determined 4 h after injection by FAAS. Data are expressed as the percentage of injected dose of platinum, together with the biodistribution of liposomes determined by ^{111}In radioactivity counting (Table 3). Higher levels of PC/Chol/G_{M1} and PC/Chol/PEG3000-PE liposomes remained in the blood as compared with PC/Chol liposomes. This was accompanied by significantly higher platinum levels in the blood for these long-circulating liposomes. It is also noteworthy that, as compared to the liposome levels in the spleen and liver, substantially lower levels of platinum were detected in these organs. In contrast, much higher platinum levels were detected in the lung and heart even though only a small fraction of liposomes accumulated in these organs. The amount of total platinum recovered in major organs was much lower with PC/Chol liposomes (44%) than PC/Chol/G_{M1} (65%) and PC/Chol/PEG3000-PE liposomes (73%).

Accumulation of NDDP-containing liposomes in RIF-1 tumor

Several studies have shown efficient accumulation of long-circulating liposomes in various solid tumors [5, 8–11]. To demonstrate that NDDP-containing long-circulating liposomes are also able to accumulate in RIF-1 solid tumor efficiently, various NDDP-containing PC/Chol-based liposomes were prepared with average diameters ranging from 117 to 136 nm and injected

Table 2 Biodistribution of NDDP-containing liposomes with various lipid compositions. ^{111}In-labelled liposomes with the indicated lipid composition and average dimeter were injected i.v. into mice at a doe of 0.4 mg lipid per mouse. Biodistribution was examined 3 h after injection. The composition of the liposomes in terms of lipid molar ratios are discribed in Materials and methods. Values are means (SD), $n = 3$

Lipid composition	Average diameter (nm)	% Injected dose				
		Blood	Spleen	Liver	Others[a]	RES/Blood
PC/Chol	147 (35)	15.0 (3.5)	23.4 (2.2)	41.2 (5.8)	2.9 (0.5)	4.3
PC/Chol + NDDP	144 (48)	23.8 (3.2)	13.2 (0.9)	37.8 (0.6)	4.4 (0.7)	2.1
PC/Chol/G_{M1}	136 (40)	50.2 (5.4)	4.4 (0.7)	20.3 (0.9)	7.4 (1.0)	0.5
PC/Chol/G_{M1} + NDDP	108 (34)	52.6 (1.6)	1.8 (0.3)	18.8 (1.7)	7.5 (1.3)	0.4
PC/Chol/PEG3000-PE	161 (49)	58.4 (4.4)	4.4 (0.5)	21.1 (1.9)	7.0 (1.6)	0.4
PC/Chol/PEG3000-PE + NDDP	167 (55)	58.3 (6.3)	3.5 (0.2)	20.3 (1.4)	7.3 (1.6)	0.4
PC/Chol/PEG1000-PE	114 (54)	28.7 (0.6)	9.2 (0.7)	38.2 (1.0)	5.5 (0.6)	1.7
PC/Chol/PEG1000-PE + NDDP	140 (50)	23.3 (1.2)	8.7 (1.4)	41.3 (5.4)	4.6 (0.5)	2.1
PC/Chol/DMPG	171 (56)	4.7 (0.8)	25.3 (2.7)	57.5 (5.5)	1.4 (0.1)	18
PC/Chol/DMPG + NDDP	212 (74)	2.2 (1.0)	23.0 (1.8)	53.9 (2.5)	1.3 (0.2)	35
DMPC/DMPG	211 (55)	8.7 (1.2)	11.9 (0.9)	61.6 (1.6)	1.5 (0.2)	8.4
DMPC/DMPG + NDDP	222 (60)	8.5 (0.9)	13.7 (1.3)	63.8 (1.7)	1.5 (0.3)	9.1

[a] 'Others' include lung, heart, and kidney

Table 3 Disposition of platinum after i.v. administration of NDDP-containing liposomes. NDDP incorporated in ^{111}In-labelled liposomes with the indicated lipid composition and average diameter or NDDP solubilized in 2% Tween 20 were injected i.v. into female C3H/HeJ mice at a dose of 0.01 mg NDDP or 0.33–0.42 mg lipid per mouse. Biodistribution of ^{111}In-labelled liposomes and platinum was examined 4 h after injection. The composition of the liposomes in terms of lipid molar ratios are described in Materials and methods. Values are means (SD), $n = 3$

Lipid composition	Average diameter (nm)		% Injected dose						Total recovery (%)
			Blood	Spleen	Liver	Lung	Heart	Kidney	
PC/Chol + NDDP	140 (44)	^{111}In	10.4 (4.4)	10.9 (1.3)	56.2 (9.8)	0.6 (0.2)	0.6 (0.4)	1.5 (0.6)	80.1 (5.5)
		Pt	15.0 (2.0)	1.7 (0.2)	7.7 (1.7)	11.3 (0.4)	6.0 (0.0)	2.0 (0.0)	43.7 (4.8)
PC/Chol/G_{M1} + NDDP	133 (47)	^{111}In	42.3 (3.3)	4.6 (0.3)	28.3 (1.8)	2.0 (0.6)	1.5 (0.3)	2.9 (0.1)	81.5 (4.1)
		Pt	27.8 (3.9)*	1.1 (0.6)	12.6 (1.3)	10.9 (0.6)	9.9 (1.8)	2.9 (0.6)	65.2 (1.7)
PC/Chol/PEG3000-PE + NDDP	134 (50)	^{111}In	38.3 (3.0)	3.7 (0.7)	32.0 (4.8)	1.4 (0.1)	1.4 (0.5)	2.9 (0.6)	79.7 (9.4)
		Pt	31.7 (0.1)**	3.5 (0.5)	12.5 (1.5)	10.6 (0.5)	9.2 (0.1)	5.2 (1.0)	72.7 (3.5)
NDDP in 2% Tween 20	–	Pt	26.6 (3.4)	0.4 (0.0)	22.8 (0.8)	3.0 (0.5)	6.0 (1.5)	2.4 (1.2)	61.0 (4.4)

*$P = 0.06$, **$P = 0.05$ vs PC/Chol + NDDP value

i.v. into RIF-1 tumor-bearing mice. The biodistribution of liposome in tumor-bearing mice was examined at 24 h after injection at which time the maximal accumulation of liposomes in tumor was normally obtained (data not shown). Data are expressed as the percentage of injected dose per organ (for the blood, spleen, and liver) or per gram of tissue (for the tumor), together with the respective RES/tumor ratio (Table 4). PC/Chol liposomes accumulated in tumor at 2.7% of the injected dose with an RES/tumor ratio of 27. PC/Chol/G_{M1} and PC/Chol/PEG3000-PE liposomes showed an enhanced accumulation of liposomes in tumor (7.5% and 8.7%, respectively) and a decreased RES/tumor ratio of 4.5 and 8.1, respectively. PC/Chol/PEG1000-PE liposomes, exhibiting a lower ability to remain in the blood circulation than G_{M1}- and PEG3000-PE-containing liposomes (Table 2), showed only a small increase in tumor accumulation (4.7%). PC/Chol/DMPG and DMPC/DMPG liposomes showed only a limited accumulation in the tumor (1.9 and 1.1%, respectively) in accordance with their rapid clearance from the blood circulation (Table 2). These results indicate that the level of liposome accumulation in tumor was directly proportional to the ability of liposomes to remain in the blood circulation.

Growth inhibition of RIF-1 tumor cells in vitro by NDDP incorporated into liposomes

Previous studies have shown that the antitumor activity of liposomal NDDP depends highly on the lipid composition of liposomes [36–38]. To assess antitumor activity of NDDP incorporated into PC/Chol-based liposomes, liposomes were prepared to include NDDP at 3.0 mol% in the lipid mixture and tested for in vitro cytotoxicity on RIF-1 tumor cells. In this study, RIF-1 tumor cells were incubated with liposomal NDDP at various concentrations (0.01–100 μM) for different drug exposure times (5, 24, and 72 h), and the fraction of viable cells was quantitated by incorporation of crystal violet. Data are expressed as the IC_{50} value for each liposomal NDDP formulation (Table 5). In general, decreasing IC_{50} values were obtained with increasing drug exposure time. Also, the cytotoxicity of NDDP appeared to vary depending on the lipid composition of the liposomes. NDDP incorporated into PC/Chol liposomes showed the lowest cytotoxicity with an IC_{50} value of 32 μM for the 5 h exposure time. Approximately a 6.8-fold increase in NDDP cytotoxicity was obtained with PC/Chol/G_{M1} liposomes ($IC_{50} = 4.7\ \mu M$). A significant increase in NDDP cytotoxicity was observed for PEG-PE-containing liposomes, PC/Chol/PEG3000-PE and PC/Chol/PEG1000-PE liposomes (52- and 42-fold increase, respectively) as compared with PC/Chol liposomes. NDDP incorporated into DMPG-containing liposomes, PC/Chol/DMPG and DMPC/DMPG liposomes, showed comparable cytotoxicity to PEG-PE-containing liposomes. These results indicate that the presence of certain amphiphiles in the liposome membranes could enhance the cytotoxicity of liposomal NDDP to varying degrees. The effect of PEG-PE and DMPG in enhancing the cytotoxicity of liposomal NDDP appeared to be comparable, while the effect of G_{M1} was much lower.

Effect of liposomal NDDP on growth rate of RIF-1 tumor in mice

The in vivo antitumor activity of liposomal NDDP was assessed using the RIF-1 tumor model [39, 40]. In this study, various NDDP-containing PC/Chol-based liposomes and DMPC/DMPG liposomes were prepared with average diameters ranging from 132 to 190 nm. The RIF-1 tumor-bearing mice were treated twice with liposomal NDDP formulations on days 12 and 16 after tumor cell inoculation. The dose of each treatment was 25 mg NDDP/kg. The increase in tumor volume of the treated mice was measured. The results are expressed as percentages in relation to tumor volume at the onset of treatment (Fig. 1). A significant decrease in the tumor

Table 4 Accumulation of NDDP-containing liposomes in RIF-1 tumor in mice. NDDP-containing ^{111}In-labelled liposomes with the indicated lipid composition and average diameter were injected i.v. into RIF-1 tumor-bearing mice at a dose of 0.4 mg lipid per mouse. Biodistribution of liposomes was examined 24 h after injection. The composition of the liposomes in terms of lipid molar ratios are described in Materials and mathods. Values are means (SD), $n = 3$. Liposome accumulation in the tumors was expressed as the percentage of injected dose per gram of tissue.

		% Injected dose				
Lipid composition	Size (nm)	Tumor	Blood	Spleen	Liver	RES/Tumor
PC/Chol + NDDP	133 (48)	2.7 (0.3)	0.8 (0.2)	13.9 (0.4)	58.9 (3.1)	27.0
PC/Chol/G_{M1} + NDDP	117 (37)	8.7 (0.3)*	15.7 (0.4)	12.6 (0.3)	35.8 (3.1)	4.5
PC/Chol/PEG3000-PE + NDDP	136 (40)	7.5 (0.1)*	1.2 (0.5)	3.7 (0.1)	48.4 (2.2)	8.1
PC/Chol/PEG1000-PE + NDDP	125 (32)	4.2 (0.7)**	0.4 (0.0)	7.7 (0.3)	56.2 (1.2)	15.2
PC/Chol/DMPG + NDDP	146 (58)	1.9 (0.3)	0.4 (0.0)	17.5 (1.0)	54.7 (1.1)	38.0
DMPC/DMPG + NDDP	182 (68)	1.1 (0.1)	0.3 (0.0)	10.9 (1.4)	70.4 (2.8)	73.9

*$P < 0.004$, **$P = 009$ vs PC/Chol + NDDP; *$P < 0.001$, **$P < 0.05$ vs PC/Chol/DMPG + NDDP and DMPC/DMPG + NDDP

Table 5 In vitro cytotoxicity of liposomal NDDP on RIF-1 cells. RIF-1 tumor cells were incubated with NDDP incorporated in liposomes with the indicated lipid composition or with NDDP solubilized in 2% Tween 20 for the indicated time interval. The composition of the liposomes in terms of lipid molar ratios are described in Materials and methods

Lipid composition	IC_{50} (μM)		
	5 h	24 h	72 h
PC/Chol + NDDP	32	28	22
PC/Chol/G_{M1} + NDDP	4.7	3.2	3.2
PC/Chol/PEG3000-PE + NDDP	0.62	0.32	0.30
PC/Chol/PEG1000-PE + NDDP	0.80	0.36	0.34
PC/Chol/DMPG + NDDP	1.3	0.52	0.40
DMPC/DMPG + NDDP	1.2	0.33	0.32
NDDP in 2% Tween 20	0.92	0.36	0.40

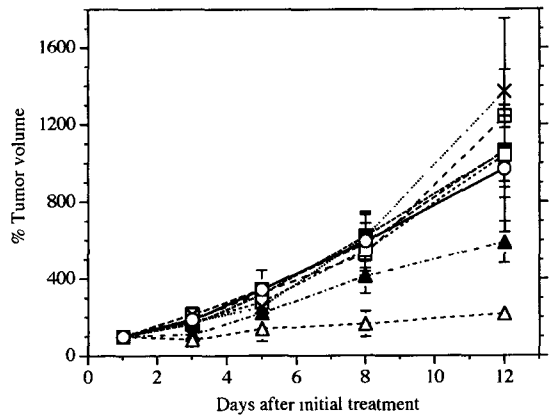

Fig. 1 Tumor growth curves of mice treated with liposomal NDDP. RIF-1 tumor-bearing mice were treated twice on days 12 and 16 after tumor cell inoculation with (-○-) PBS, (-✳-) NDDP solubilized in 2% Tween 20 or NDDP incorporated into (-□-) PC/Chol (132 nm), (-■-) PC/Chol/G_{M1} (180 nm), (-△-) PC/Chol/PEG3000-PE (144 nm), (--▲--) PC/Chol/PEG1000-PE (144 nm), (-⊠-) PC/Chol/DMPG (155 nm), and (-⊞-) DMPC/DMPG (190 nm) at a dose of 25 mg NDDP per kg body weight. Data are expressed as the percentage of tumor volume relative to the tumor volume at onset of treatment and plotted as a function of time after the initial treatment. Bars represent SD ($n = 5$)

growth rate was observed when NDDP was formulated in PC/Chol/PEG3000-PE and PC/Chol/PEG1000-PE liposomes, the former showing the higher antitumor effect. Other liposomal formulations, including PC/Chol, PC/Chol/G_{M1}, PC/Chol/DMPG, and DMPC/DMPG liposomes, did not show any significant effect on the tumor growth rate.

Discussion

A lipophilic prodrug, NDDP, formulated in liposomes has been extensively studied for potential applications in cancer therapy [32–35]. These studies utilized large (1–5 μm) multilamellar liposomes composed of DMPC and DMPG, a liposome formulation exhibiting a high affinity to the RES organs. This formulation has been also shown to facilitate the intraliposomal activation of NDDP [35–38]. Thus, DMPC/DMPG liposomes deliver a high level of the prodrug to the RES organs, at which the prodrug can be converted to a fully active species for antitumor action. This formulation is especially effective for the therapy of cancer residing in the RES organs such as the liver. However, therapy for tumors in non-RES organs is severely limited with this formulation. Several studies have indicated that liposomes with reduced affinity to the RES and prolonged circulation times could provide a system suitable for targeting anticancer drugs to non-RES organs [5, 8–11]. In the present study, we tested G_{M1}- and PEG-PE-containing long-circulating liposomes in an attempt to develop a liposomal NDDP formulation for targeting to non-RES organs. Specifically, we examined, using PC/Chol liposomes as a basic liposome formulation, the effect of G_{M1} and PEG-PE incorporation on the biodistribution, in vitro cytotoxicity, and in vivo antitumor activity of NDDP-containing liposomes. In these studies, the RIF-1 fibrosarcoma model was used as a non-RES target organ. This tumor model has been widely used in studies of experimental therapeutics [39, 40], including ones with cisplatin [50, 51].

Since the biodistribution profile of liposomes could be altered by the presence of an additional lipid component in the liposome membranes, we first examined the effect of NDDP incorporation on the liposome biodistribution. Data in Table 2 show that the activity of G_{M1} and PEG-PE in prolonging the circulation times of liposomes is preserved in the presence of 3.0 mol% NDDP in the lipid mixture. This is an expected result since incorporation of NDDP did not significantly alter the surface hydrophilicity of the liposomes (Table 1). Thus, the pharmacokinetic profile of NDDP is also expected to be altered by incorporation of the prodrug molecules into G_{M1}- or PEG-PE-containing liposomes. In fact, significantly higher platinum levels in the blood were detected when NDDP was incorporated into these long-circulating liposomes as compared to the conventional PC/Chol liposomes (Table 3). It was also noted that platinum levels in the liver and spleen were significantly lower than those of liposome accumulation. In contrast, substantial amounts of platinum were found in the lung and heart, whereas only a small fraction of liposomes were detected in these organs. These observations indicate that liposomal NDDP concentrated in the liver and spleen undergoes a rapid intracellular metabolism at these organs, followed by a redistribution of NDDP-derived metabolites to the lung and heart. It should be noted that the total recovery of platinum was significantly lower for PC/Chol liposomes than for PC/Chol/G_{M1} and PC/Chol/PEG3000-PE liposomes. Presumably, PC/Chol liposomes exhibited a higher rate of liver accumulation, and thus the rate of intracellular metabolism

in the liver might also have been high. Fractions of the metabolites may be redistributed to other organs or excreted, thus resulting in a relatively low recovery of platinum. The rate of liposome accumulation in the liver was much slower for PC/Chol/G_{M1} and PC/Chol/PEG3000-PE liposomes, thus resulting in the higher platinum levels in the blood as well as a higher recovery of platinum.

The mechanism of liposome accumulation in tumors has been examined in several studies [52, 53]. It has been shown that certain solid tumors exhibit a significantly higher microvascular permeability than normal organs [6, 7]. Given the high liposome concentrations in the blood, long-circulating liposomes have an increased chance of extravasation into the tumor. Data in Table 4 show efficient accumulations of PC/Chol/G_{M1} and PC/Chol/PEG-PE liposomes in the tumor as compared to the conventional PC/Chol liposomes. The highest tumor accumulation was obtained for liposomes containing G_{M1} or PEG3000-PE (Table 4), amphiphiles having comparable activity in prolonging the circulation times of liposomes (Table 2). PEG1000-PE, having a lower activity in prolonging the circulation times of liposomes than G_{M1} and PEG3000-PE, also enhanced liposome accumulation but at a level lower than G_{M1}- and PEG3000-PE-containing liposomes. These results indicate that the ability of liposomes to accumulate in tumors was directly proportional to their ability to remain in the blood circulation. Taken together with the data in Table 3 showing the high levels of platinum in the blood circulation when long-circulating liposomes were used as carriers, it can be expected that increased amounts of the prodrug or drug molecules are delivered to the tumor.

NDDP requires a chemical conversion to exert its biological function. Previous studies suggest that the presence of DMPG in the liposome membranes facilitates the activation of NDDP [36–38]. Data in Table 4 show that the presence of PEG-PE in liposome membranes also enhanced the cytotoxicity of NDDP incorporated into PC/Chol-based liposomes. The activity of PEG-PE in enhancing the cytotoxicity of liposomal NDDP appeared to be comparable to that of DMPG, as shown by the similar IC_{50} values of NDDP when formulated in PC/Chol/PEG3000-PE and PC/Chol/DMPG liposomes (Table 5). Previously, NDDP formulated in DMPC/DMPG liposomes has been shown to exhibit a decreased stability as indicated by low levels of intact NDDP at specified times after liposome preparation. It has also been shown that there is an inverse relationship between the antitumor activity and stability of liposomal NDDP. This finding led to the concept of 'intraliposomal prodrug activation' [36], in which the prodrug is converted into the active species in the liposome membrane at a rate determined by the lipid composition. Interestingly, we found that incorporation of PEG-PE into PC/Chol liposomes also resulted in reduced stability of NDDP. For example, the fraction of intact NDDP after 24 h in the liposome preparation, measured by HPLC, was only 29% when formulated in PC/Chol/PEG3000-PE liposomes, as compared to 78% for PC/Chol liposomes. These observations suggest that both PEG-PE and DMPG enhance the cytotoxicity of liposomal NDDP by lowering the drug stability, although detailed mechanisms have yet to be determined.

Data in Fig. 1 show that NDDP was effective in reducing the tumor growth rate only when incorporated into PC/Chol/PEG-PE liposomes, a formulation supporting both efficient accumulation in the tumor (Table 4) and enhanced cytotoxicity of liposomal NDDP (Table 5). PC/Chol/DMPG and DMPC/DMPG liposomes, both of which enhanced the cytotoxicity of liposomal NDDP (Table 5) but did not show a high level of tumor accumulation (Table 4), were not effective in inhibiting tumor growth. Furthermore, NDDP formulated in PC/Chol/G_{M1} liposomes, showing enhanced tumor accumulation (Table 4), but exhibiting much lower cytotoxicity than PC/Chol/PEG-PE liposomes, were not effective either. These results indicate that therapy with liposomal NDDP of solid tumors residing outside the RES organs requires two independent functions on the liposomes: the ability to remain in the blood circulation for prolonged periods of time and to enhance the intraliposomal prodrug activation. PEG-PE is unique in providing both functions. PEG-PE-containing liposomal NDDP formulations described here deserve further evaluation for therapeutic applications.

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