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Comparison of different hydrophobic anchors conjugated to poly(ethylene glycol): effects on the pharmacokinetics of liposomal vincristine

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Abstract

Poly(ethylene glycol) (PEG) conjugated lipids have been used to increase the circulation longevity of liposomal carriers encapsulating therapeutic compounds. PEG is typically conjugated to distearoylphosphatidylethanolamine (DSPE) via a carbamate linkage that results in a net negative charge on the phosphate moiety at physiological pH. It was anticipated that the presence of this negative charge could have deleterious effects on liposome pharmacokinetic characteristics. We describe here the synthesis of a new class of neutrally charged PEG-lipid conjugates in which the PEG moiety was linked to ceramide (CER). These PEG-CER conjugates were compared with PEG-DSPE conjugates for their effects on the pharmacokinetics of liposomal vincristine. PEG-CER (78% palmitic acid, C16) and PEG-DSPE achieved comparable increases in the circulation lifetimes of sphingomyelin/cholesterol (SM/chol) liposomes. However, PEG-DSPE significantly increased the in vitro and in vivo leakage rates of vincristine from SM/chol-based liposomes compared to vincristine leakage observed when PEG-CER was used. The increase in drug leakage observed in vitro that was due to the presence of PEG-DSPE was likely due to the presence of a negative surface charge. Analysis of the electrophoretic mobilities of these formulations suggested that the negative surface charges were shielded by approx. 80% by the PEG layer extending from the membrane surface. In contrast, formulations containing PEG-CER had no surface charge and no electrophoretic mobility. A comparison of the effects of the ceramide acyl chain length (C8 through C24) on the pharmacokinetics of SM/chol/PEG-CER formulations of vincristine demonstrated that longer acyl chains on the PEG-CER were associated with longer circulation lifetimes of the liposomal carriers and, consequently, higher plasma vincristine concentrations. These data suggest that the short chain PEG-ceramides underwent rapid partitioning from the vesicles after i.v. administration, whereas the longer chain PEG-ceramides had stronger anchoring properties in the liposome bilayers and partitioned slowly from the administered vesicles. These data demonstrate the utility of ceramide-based steric stabilizing lipids as well as the potential for developing controlled release formulations by manipulating the retention of the PEG-ceramide conjugate in liposome bilayers.

Keywords: Vincristine; Liposome; Poly(ethylene glycol); Pharmacokinetics; Ceramide; Phosphatidylethanolamine

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1. Introduction

Liposomes have been extensively characterized as delivery vehicles that effectively prolong the circulation longevity of a wide variety of therapeutic agents after parenteral administration, reduce drug toxicity and increase drug accumulation at disease sites. It is widely accepted for many therapeutic agents that increases in the drug's circulation longevity is associated with increased therapeutic activity. To this end, modifications to liposomal carriers that increase their circulation lifetime have been reported to cause substantial increases in the therapeutic activity of the drug [1,2]. Typically, these modifications have been achieved by incorporating poly(ethylene glycol) (PEG) that has been conjugated to phosphatidylethanolamine (PE) in the liposomal membrane [3]. While a variety of different PEG sizes may be conjugated to the phospholipid, and conjugation may be achieved with many distinct chemical linkages [4], the common feature of these approaches is the use of PE (typically distearyol-PE, DSPE) as the lipid anchor. Further, most commercially available PEG-PE is synthesized with a carbamate linkage that results in a net negative charge on the phosphate headgroup of the conjugate at physiological pH [5].

We anticipated that the incorporation of a non-charged PEG-lipid yielding a neutral surface electrostatic charge would increase the circulation lifetimes of liposomes beyond that achieved using PEG-PE. Negative liposome surface charge has been closely associated with rapid clearance from the circulation [6] and both negative surface potentials and electrophoretic mobility are present in liposomes containing PEG-PE [5]. It was also anticipated that a reduction in the negative surface charge of the liposomes in the presence of PEG-lipids might reduce the leakage rates of encapsulated cationic drugs since the neutral bilayer surface will not attract positively charged drug to the membrane surface. To achieve these characteristics, we have chosen to conjugate PEG to ceramides using linkage chemistry that results in a neutral, non-charged molecule at physiological pH.

Given the increased drug leakage observed for sphingomyelin/cholesterol formulations of vincristine when PEG-DSPE was incorporated as a steric-stabilizing lipid [7], we utilized liposomal vincristine as a model system to evaluate the effect of membrane surface charge on drug retention. In this report, we compare PEG-DSPE and PEG-CER containing liposomes for their in vivo pharmacokinetics and drug retention characteristics. In addition, we examine the influence of the hydrophobic anchor on circulation longevity and drug retention for neutral PEG-ceramides having a variety of acyl chain lengths.

2. Materials and methods

2.1. Materials

Egg sphingomyelin (SM) and D-sphingosine were purchased from Avanti Polar Lipids and used without further purification. Cholesterol (chol) was obtained from Sigma (St. Louis, MO, USA) and poly(ethylene glycol)$_{2000}$-distearoylphosphatidylethanolamine (PEG-DSPE) from Northern Lipids (Vancouver, B.C., Canada). Vincristine sulfate (Oncovin) was obtained from Eli Lilly (Canada). Cholesteryl-4-hexadecyl ether (CHDE) radiolabeled with ^{14}C was obtained from New England Nuclear. [^{3}H]Vincristine was obtained from Amersham (Canada). All other chemicals were obtained from Sigma (St. Louis, MO, USA). Female BDF_1 mice (8–10 weeks old) were obtained from Charles River Laboratories.

2.2. Synthesis of $MePEG_{2000}$-S

Monomethoxypolyethylene glycol (average MW = 2000; $MePEG_{2000}$) (4 g) was dissolved in 30 ml of CH_2Cl_2 and treated with 600 mg of succinic anhydride, 400 mg triethylamine and 250 mg 4-dimethylaminopyridine (DMAP) by stirring under nitrogen gas for 16 h at 20°C. The reaction solution was diluted with 60 ml of CH_2Cl_2 and then cooled on ice and 50 ml of water was added. This mixture was acidified with 1 N HCl and the organic layer recovered. The aqueous layer was further extracted twice with 30 ml of CH_2Cl_2 and the combined organic extracts were dried. The crude product was purified on silica gel (G60) column eluted with CH_2Cl_2 containing 2–8% methanol. Column fractions were analyzed by thin layer chromatography (TLC) (silica gel, $CHCl_3/CH_3OH$ 88:12, v/v) and fractions containing the pure product of $MePEG_{2000}$-S ($R_f = 0.4$) were pooled and concen-

trated. Trituration with diethyl ether yielded 3.2 g of white $MePEG_{2000}$-S product.

2.3. Synthesis of C8-, C14- and C20-ceramides

N-Hydroxysuccinimide (NHS) esters of octanoic acid, myristic acid, eicosanoic acid and tetracosanoic acid were synthesized as described previously [8]. One mmole of each ester was added to a solution of 300 mg of D-sphingosine in 16 ml of anhydrous CH_2Cl_2 and containing 118 mg of triethylamine. The mixture was stirred under nitrogen gas for 4 h at 20–30°C. Analysis by TLC (silica gel, $CHCl_3/CH_3OH/H_20$ 65:25:4 v/v/v or $CHCl_3/CH_3OH$ 9:1 v/v) indicated that most of the D-sphingosine had reacted. The reaction mixture was cooled on ice and diluted with 60 ml of CH_2Cl_2, 30 ml of H_2O and then neutralized with 1 N HCl. The CH_2Cl_2 layer was washed twice with 30 ml of H_2O and then dried in vacuo. The residue was recrystalized twice from acetone to yield the pure product.

2.4. Synthesis of $MePEG_{2000}$-S-ceramides

The egg ceramide used here was obtained from Avanti Polar Lipids and derived from egg sphingomyelin. This egg ceramide had the amide composition of egg sphingomyelin and was comprised predominantly of palmitic acid (78 mol% of 16:0 and 7 mol% of 18:0, with the remainder comprised of 2–4 mol% of 20:0, 22:0, 24:0 and 24:1) and will be referred to as PEG-CER (C16) here. The appropriate ceramide (egg ceramide or specific fatty acyl ceramides synthesized as above) were mixed with dicyclohexylcarbodiimide (DCC) and DMAP in 6–8 ml of warm anhydrous CH_2Cl_2 in weight ratios of ceramide/DCC/DMAP approx. 4.5:2:1. $MePEG_{2000}$-S in 2 ml of anhydrous CH_2Cl_2 was added dropwise to the above solution, at a $MePEG_{2000}$-S/ceramide weight ratio of approx. 4:1, and stirred under nitrogen gas for 6 h at room temperature. Any precipitated dicyclohexylurea was filtered off and the filtrate was concentrated in vacuo. Trituration of the solid residue with diethyl ether removed most of the DCC, DMAP and unreacted ceramide. The crude product was chromatographed on a short silica gel column (G60) eluted with CH_2Cl_2/CH_3OH (92:2, v/v) and the fractions containing the product were combined, evaporated to dryness then dissolved in distilled water and dialyzed at 4°C overnight against distilled water. Lyophilization of the product yielded a white powder which was detected as a single spot after TLC analysis ($R_f = 0.5$ on silica gel in $CHCl_3/CH_3OH$ 9:1, v/v).

2.5. ^1H-Nuclear magnetic resonance

The initial ceramide, initial $MePEG_{2000}$-S and the final PEG-ceramide products were dissolved in $CDCl_3$ and analyzed by ^1H-nuclear magnetic resonance (^1H-NMR) using a Bruker 400 MHz instrument using residual chloroform (δ_H 7.24) as the reference.

2.6. Liposome preparation and vincristine loading

The preparation of liposomes and their loading with vincristine has been described in detail previously [7]. Briefly, lipid films containing 2–5 μCi [^{14}C]CHDE were prepared by the drying of lipid solutions in chloroform/methanol under high vacuum. These lipid films were hydrated at 100 mg lipid/ml in 0.3 M citrate (pH 4.0) to produce multilamellar vesicles. Large unilamellar vesicles were obtained by repeated extrusion of the hydrated lipids through two stacked 0.1 μm filters (Poretics, Livermore, CA, USA) held at 60–65°C in a Thermobarrel extruder (Lipex Biomembranes, Vancouver, Canada). Liposome mean diameters, confirmed by quasi-elastic light scattering using a Nicomp 270 or Nicomp T370 particle sizer, were typically 120–130 nm. The uptake of vincristine into these vesicles was accomplished in response to a transmembrane pH gradient [9]. A solution of vincristine, containing [^3H]-vincristine, was added to the liposomes to achieve a final drug/lipid ratio of 0.1:1 (w/w). A transmembrane pH gradient was generated by the addition of sufficient 0.5 M disodium hydrogen phosphate to bring the external pH to 7.2–7.6. Vincristine uptake was allowed to proceed for 10 min at 60°C. Uptake efficiencies were checked by chromatography of aliquots of the loaded liposomes on a Sephadex G-50 column and were typically greater than 95%.

2.7. In vitro release of vincristine from liposomes

Liposomal vincristine was prepared as described above. After vincristine loading, the preparations were diluted 1:1 with mouse serum (Sigma) then transferred into a dialysis tubing (Spectra/Por, molecular weight cutoff = 3500). Samples were dialyzed against 1500 vols. of 150 mM NaCl, 20 mM HEPES (pH 7.5) at 37°C for up to 72 h. At various times, aliquots were taken from the dialysis tubing then passed over a Sephadex G-50 column. Eluates were analyzed by liquid scintillation counting (LSC) for quantitation of lipid and drug.

2.8. Electrophoretic mobility measurements

Lipid mixtures were prepared in chloroform/methanol as described above, but without [^{14}C]CHDE, then rehydrated in 10 mM sodium chloride (pH 7.0). Each dispersion of multilamellar vesicles was placed in a cylindrical microelectrophoresis chamber (Rank Mark I) and an electric field of 3.9 V/cm was applied at a temperature of 25°C. Vesicle migration in the electric field was determined by timing vesicle passage through a pre-determined number of squares that were viewed at a magnification of 40×. The largest in-focus particles were examined and no size dependence was observed. Each preparation was measured 20 times, accomplished by ten measurements in each direction with the polarities switched between timings. The electrophoretic mobility was calculated from the averaged velocities, the applied voltage and the chamber electrical length. Fixed red blood cells had a mobility of -1.2 (± 0.1) cm^2/Vs in 0.10 M NaCl.

2.9. Pharmacokinetics of liposomal vincristine formulations

Liposomal formulations of vincristine prepared as described above using [^{3}H]vincristine and [^{14}C]CHDE as a lipid label. The formulations were diluted to the appropriate concentrations for administration using 150 mM NaCl, 20 mM HEPES (pH 7.5) and administered to female mice by lateral tail vein injection at a vincristine dose of 2.0 mg/kg (corresponding to a lipid dose of 20 mg/kg). At various times after liposome administration, blood was recovered by cardiac puncture from three animals per treatment group. Lipid and vincristine quantities in the plasma were assayed by LSC.

3. Results

3.1. Characterization of PEG-ceramides

The chemical structure of the PEG-ceramides is shown in Fig. 1. All synthetic products were analyzed by TLC and gave single spots at the expected R_f values for the pure products. In addition, the final products were analyzed by ^{1}H-nuclear magnetic res-

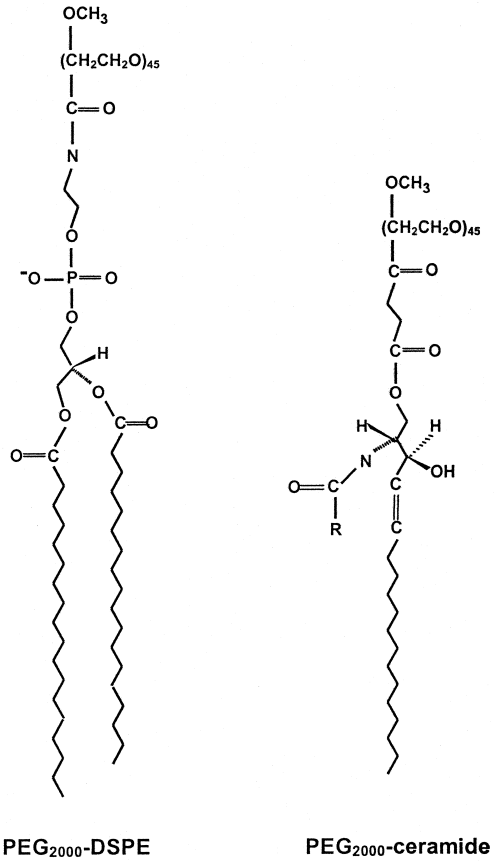

Fig. 1. Chemical structures of PEG-DSPE and PEG-ceramides. The structure for PEG_{2000}-DSPE is modified from that provided by Avanti Polar Lipids and it is assumed that 45 poly(ethylene glycol) groups are used to comprise a 2000 Da PEG moiety. For the PEG-ceramides, the R group was C_7H_{15} (for the C8-ceramide), $C_{13}H_{27}$ (for the C14-ceramide), $C_{15}H_{31}$ (78 mol%; for the egg ceramide), $C_{19}H_{39}$ (for the C20-ceramide) or $C_{23}H_{47}$ (for the C24-ceramide).

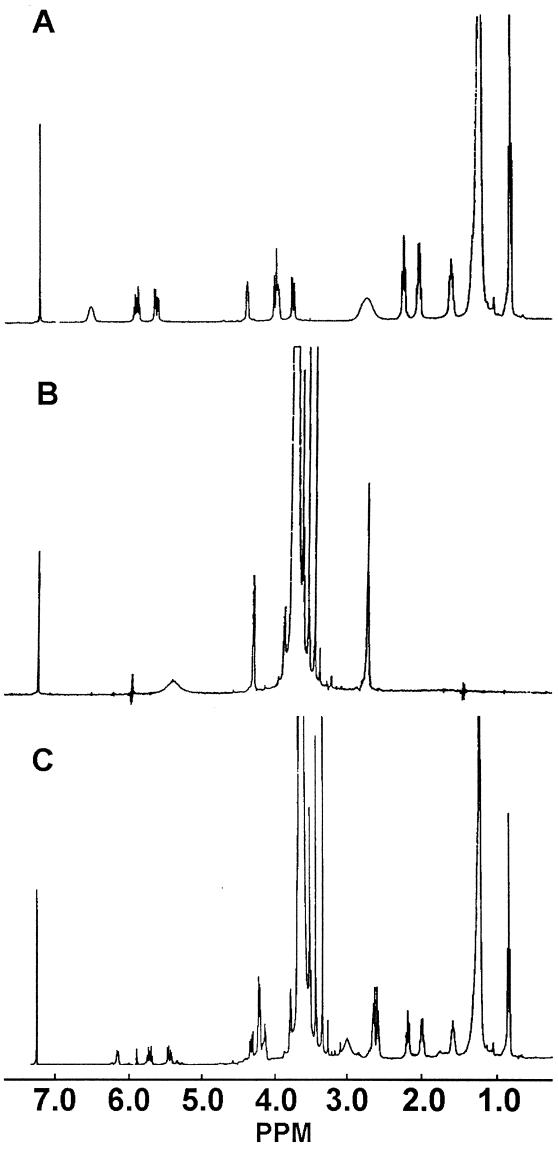

Fig. 2. ^{1}H-NMR spectra of ceramide (A), $MePEG_{2000}$-S (B) and PEG-ceramide (C8) (C).

onance to confirm that the products had the expected structure. Data for PEG-ceramide (C8) are presented in Fig. 2 by way of example. The complete characterization of the PEG-ceramides is to be presented in a separate paper (Wang et al., manuscript in preparation).

For PEG-ceramide (C8), the methyl and methylene proton resonances between 0.8 and 2.5 ppm clearly arose from the ceramide (C8) starting material (compare Fig. 2C with A). Similarly, resonances between 5.5 and 6.5 ppm in the PEG-ceramide material (Fig. 2C) were also derived from the ceramide (C8) starting material (Fig. 2A). The complex of resonances in the range of 3.0–4.0 ppm arose from the PEG polymer protons (Fig. 2B). The specificity of the conjugation to the hydroxyl of the primary carbon of the ceramide is shown by the downfield shift of the methylene protons from 3.92 ppm in the starting ceramide (Fig. 2A) to 4.12 ppm in the final PEG-ceramide product (Fig. 2C). This shift was due to the primary ester linkage of the PEG moiety. In contrast, the proton on the tertiary carbon, and adjacent to the second hydroxyl group to which PEG might conjugate, had a resonance at 4.3 ppm in the starting ceramide (C8) (Fig. 2A) that remained at 4.3 ppm in the PEG-ceramide (C8) product (Fig. 2C). Since the downfield shift of this proton was not affected by PEGylation of the ceramide, these data indicate that the conditions used for the coupling of PEG_{2000}-S with ceramide yielded predominantly the $MePEG_{2000}$-S-ceramide product attached via the primary ester linkage.

3.2. *In vitro characterization of liposomal formulations containing PEG-CER or PEG-DSPE*

A variety of liposomal formulations of vincristine were evaluated for drug loading and subsequent drug retention during dialysis in the presence of mouse serum. Encapsulated vincristine was very well retained in SM/chol liposomes, with a half-life for drug release of 219 h (Table 1). The addition of the anionic lipid PEG-DSPE to SM/chol liposomes decreased the retention half-life of vincristine from 219 h to 101 h, an increase in leakage rate of 117% (Table 1). In contrast, identical proportions of the neutral PEG-CER induced only a 40% increase in drug leakage rate (Table 1). Since the PEG-DSPE molecule has a net negative surface charge, we examined the effects of naturally occurring anionic phospholipids on the retention of vincristine in SM/chol liposomes. The addition of 5 mol% of anionic lipids DSPG or DOPS decreased the half-life of vincristine retention to 115 h and 62 h, respectively. These decreases in half-life represent increases in vincristine leakage rates of 90% and 253% respectively and are comparable to that effected by PEG-DSPE.

To better understand the physical characteristics

of these liposomes that may be contributing to changes in vincristine retention and/or liposome circulation lifetimes, we examined the electrophoretic mobilities of several formulations in an electric field (Table 1). The neutral SM/chol liposomes and SM/chol containing 5 mol% of the neutral PEG-CER had no movement in an applied electric field (Table 1). The presence of 5 mol% of either anionic phospholipid DSPG or DOPS resulted in a substantial negative electrophoretic mobility of approx. -4.2×10^{-4} cm^2/Vs (Table 1). The anionic lipid PEG-DSPE, although carrying a net negative charge on its headgroup similar to that of DSPG and DOPS, conferred only a modest negative electrophoretic mobility of -0.83×10^{-4} cm^2/Vs, a value 80% lower than that observed with an identical molar proportion of DOPS or DSPG. The electrophoretic mobility of the SM/chol/PEG-DSPE formulation was significantly different from that of the SM/chol/PEG-CER formulation.

3.3. Pharmacokinetic comparison of PEG-CER with PEG-DSPE in liposomal vincristine

The preceding analysis of SM/chol liposomes containing PEG-DSPE or PEG-CER indicated that the electrophoretic mobilities (or their directly related zeta potentials) of these two formulations were slightly, but significantly, different (Table 1). Since a negative surface charge on liposomes is associated with rapid liposome clearance after administration [6], the different electrophoretic mobilities on SM/chol liposomes containing PEG-DSPE and PEG-CER suggested that these formulations might also have different circulation lifetimes. The addition of 5 mol% of either PEG-DSPE or PEG-CER (C16) to SM/chol formulations of vincristine increased both the plasma concentrations and the circulation lifetimes of the liposomes (Fig. 3A). As a result, the plasma lipid concentrations at 24 h after i.v. administration were comparable, between 153 (±13) and 185 (±8) μg lipid/ml plasma in the PEG-CER and PEG-DSPE containing formulations, respectively, compared to 116 (±9) μg lipid/ml plasma for SM/chol (Fig. 3A). In addition, PEG-CER and PEG-DSPE conferred identical increases in the circulation lifetimes of conventional DSPC/chol based liposomes when added at 5 mol% (data not shown).

A negative surface charge on liposomes conferred by the presence of the anionic lipids DOPS and DSPG was associated with increased vincristine leakage (Table 1). Similar increases in drug leakage were induced in vitro by the presence of PEG-DSPE whereas the neutrally charged PEG-CER had only modest effects on vincristine release from the liposomes (Table 1). However, in vivo the addition of 5 mol% of either PEG-DSPE or PEG-CER (C16) to SM/chol-based formulations was associated with significantly reduced vincristine retention, as shown by lower vincristine/lipid ratios compared to SM/chol formulations, at all times between 1 and 24 h after i.v. administration (Fig. 3B). Calculation of the half-lives of drug release from these liposomal carriers in vivo demonstrated that the rate of vincristine leakage was greater from the SM/chol/PEG-DSPE formulation than either the SM/chol/PEG-CER (C16) or SM/chol formulations (Fig. 3B). Specifically, the $t_{1/2}$ values for drug leakage were 29.1 and 33.0 h

Table 1
Summary of the in vitro characteristics of various liposomal formulations

Formulation (mol/mol)	In vitro vincristine leakage		Electrophoretic mobility ($\times10^{-4}$ cm^2/Vs)
	$t_{1/2}$ (h)	r^2	
SM/chol (55:45)	219	0.96	0.0
SM/chol/DSPG (50:45:5)	115	0.99	−4.18 (±0.25)
SM/chol/DOPS (50:45:5)	62	0.98	−4.24 (±0.31)
SM/chol/PEG-DSPE (50:45:5)	101	0.96	−0.83 (±0.05)
SM/chol/PEG-CER (C14) (50:45:5)	156	0.99	0.0

The in vitro vincristine leakage $t_{1/2}$ values were calculated from $0.693/-k$, where k is the slope of the time vs. ln(% drug/lipid ratio) plot [7] during dialysis in 50% mouse serum. The correlation coefficient (r^2) for these plots is also given. Electrophoretic mobilities were obtained from multilamellar dispersions of the lipid formulations in the absence of encapsulated vincristine as described in Section 2.

for the SM/chol and SM/chol/PEG-CER formulations compared to 23.9 h for SM/chol/PEG-DSPE (r^2 values for these regressions of the ln % D/L vs. time plots were 0.99, 0.88 and 0.85, respectively). This represents a 22% increase in the vincristine leakage rate by the addition of PEG-DSPE to SM/chol liposomes. A very similar decrease of the in vivo $t_{1/2}$ for vincristine retention from 33.3 h for SM/chol to 26.8 h for SM/chol/PEG-DSPE, representing a 24% increase in drug release rate, has been reported previously [7]. The alterations in carrier circulation lifetimes due to the presence of either PEG-DSPE or PEG-CER (C16) in SM/chol liposomes (Fig. 3A) were counteracted by the alterations in vincristine retention in these carriers such that the overall vincristine levels in the plasma were not affected by the presence of either PEG-conjugated lipid (Fig. 3C). The calculated $t_{1/2}$ values for vincristine lifetime in the circulation were 9.2, 10.9 and 10.6 h for the SM/chol, SM/chol/PEG-DSPE and SM/chol/PEG-CER formulations, respectively.

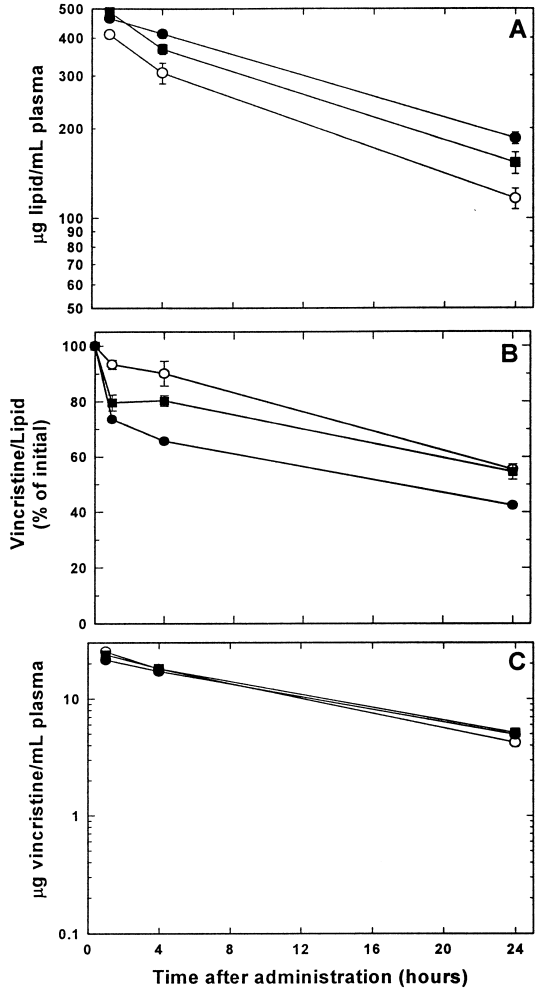

Fig. 3. Pharmacokinetic analysis of SM/chol (55:45, mol/mol) (○), SM/chol/PEG-DSPE (50:45:5, mol/mol/mol) (●) and SM/chol/PEG-CER (C16) (50:45:5) (■) formulations of vincristine. (A) Concentrations of liposomal lipid in the plasma at various times after i.v. administration. (B) Vincristine/lipid ratio (% of the administered vincristine/lipid ratio) in the plasma at various times after i.v. administration. (C) Concentrations of vincristine in the plasma at various times after i.v. administration. Data represent the mean (±S.E.) of four animals.

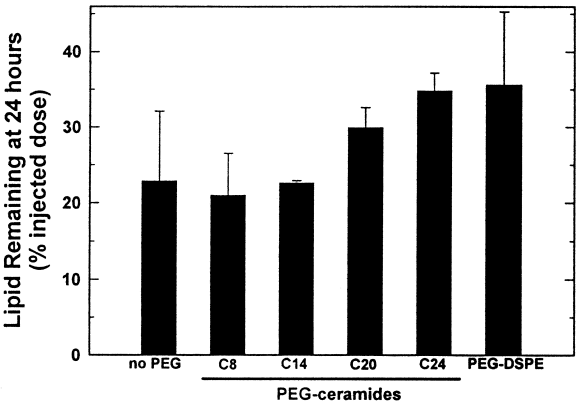

Fig. 4. Comparison of the effect of different PEG-conjugated lipids on circulation longevity of SM/chol formulations of vincristine. Data represent the proportions of the injected lipid dose (20 mg/kg) remaining in the circulation at 24 h after i.v. administration for SM/chol formulations without a PEG-conjugated lipid (no PEG) or containing 5 mol% of either PEG-DSPE or PEG-CER with differing acyl chain lengths (C8, C14, C20, C24). Data represent the mean (±S.E.) of three animals.

3.4. *Effect of PEG-CER acyl chain length on liposomal vincristine pharmacokinetics*

Since PEG-modified lipids are amphiphiles possessing large (approx. 2000 Da) hydrophilic moieties and a hydrophobic carbon chain moiety that acts to anchor the molecule in the bilayer, it was anticipated that the propensity of the PEG-conjugated lipids to partition out of the bilayer could be regulated by alterations in the balance of hydrophilic and hydrophobic domains. In practice, we have effected changes in the hydrophilic/hydrophobic balance via modifications to the carbon chain length of the hydrophobic anchor. Consequently, we have examined SM/chol formulations of vincristine containing 5 mol% of PEG-ceramide having different acyl chain

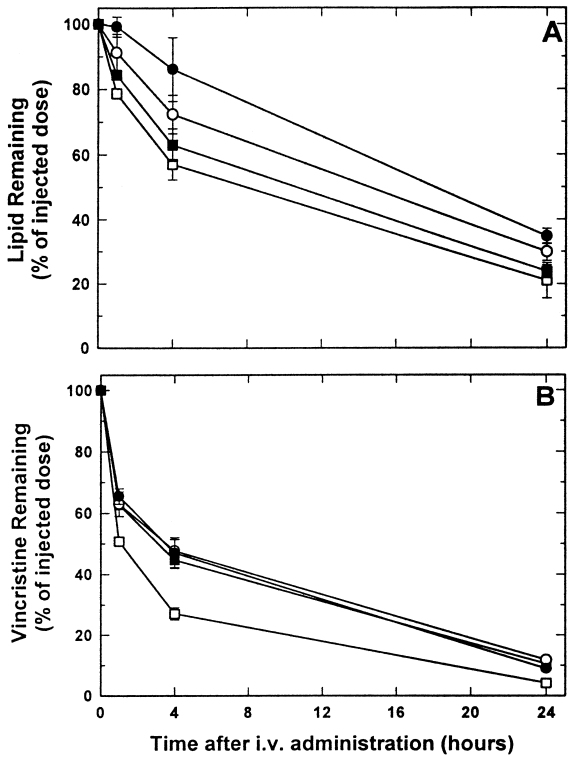

Fig. 5. Pharmacokinetic analysis of SM/chol (55:45, mol/mol) (■) and SM/chol/PEG-CER (50:45:5, mol/mol/mol) formulations of vincristine prepared using ceramide C8 (□), C20 (○), or C24 (●). (A) Liposomal lipid remaining in the plasma at various times after i.v. administration. (B) Vincristine remaining in the plasma at various times after i.v. administration. Data for SM/chol were recalculated from data presented in Fig. 1. Data represent the mean (±S.E.) of three animals.

lengths of a defined molecular species. No significant increases in the plasma concentration of liposomes 24 h after i.v. administration were observed for liposomes containing either C8 or C14 PEG-CER when compared to SM/chol vesicles without PEG (Fig. 4). However, the proportions of the injected liposomes remaining in the circulation after 24 h were significantly increased by the use of the longer-chain C20 and C24 PEG-CER (Fig. 4). Specifically, the plasma concentrations of lipid were increased by 42% and 66% in the C20 and C24 formulations, respectively, above those observed for the C8 PEG-CER formulation. The circulation lifetimes achieved with PEG-DSPE were very similar to those achieved with identical proportions of the C24 SM/chol/PEG-CER.

A more rigorous pharmacokinetic analysis was performed on these liposomal vincristine preparations. The formulation containing the short-chain C8 PEG-CER underwent a more rapid initial clearance phase followed by a slower clearance rate at times >4 h post administration (Fig. 5A). Consistent with the single time-point data presented in Fig. 4, the clearance kinetics of the C8 PEG-CER formulation was very similar to the SM/chol formulation without PEG-CER (Fig. 5A). In contrast, the formulation containing the long-chain C24 PEG-CER did not have a rapid initial clearance phase, and the formulation with the intermediate chain length C20 PEG-CER had clearance kinetics intermediate between the C8 and C24 formulations (Fig. 5A). The estimated half-lives of the liposomes in the circulation increased with increases of the acyl chain length, from 12.7 h and 11.1 h to 14.8 h and 15.2 h for the SM/chol formulations containing C8, C14, C20 and C24 PEG-CER, respectively.

For these SM/chol liposomes containing vincristine, the presence of the different chain length PEG-ceramides had only small effects on the retention of drug in vivo. In the period between 4 and 24 h post administration, the leakage of drug was identical in all formulations. Only the C8 PEG-CER formulation displayed faster vincristine leakage rates in the period between 1 and 4 h post administration (data not shown). As a consequence of the liposome circulation lifetimes and these drug retention characteristics, the vincristine concentrations in the plasma were very similar in the formulations containing C20 or C24 chain length PEG-CER or lacking PEG-CER (Fig. 5B). In contrast, SM/chol formulations containing C8 PEG-CER had significantly lower plasma concentrations of vincristine, than observed for carriers without PEG-CER or with 5 mol% of either C20 PEG-CER or C24 PEG-CER (Fig. 5B).

These data indicate that the different chain length PEG-CER had different effects on liposome and drug pharmacokinetics. For example, the two formulations having rapid and similar lipid clearance from the circulation (SM/chol and C8 SM/chol/PEG-CER; Fig. 5A) had the highest (SM/chol) and lowest (C8 SM/chol/PEG-CER) retention of vincristine in vivo. In comparison to the long chain length C20 and C24 PEG-CER formulations, the short chain length C8 PEG-CER had more rapid liposome clearance from the circulation (Fig. 5A) and more rapid drug leakage (not shown) resulting in the most rapid

drug clearance from the circulation (Fig. 5B). Overall, these data demonstrate the potential of using PEG-CER having regulated partition rates to achieve specific liposome and drug pharmacokinetics.

4. Discussion

The anti-tumor activity of vincristine in a variety of preclinical murine and human xenograft tumor models is strongly correlated with increased vincristine circulation lifetimes and increased vincristine accumulation at tumor sites [10]. The dramatic increases in vincristine circulation lifetime and accumulation at tumor sites achieved through vincristine encapsulation in liposomal carriers [9,11–14], have been accomplished primarily through increased vincristine retention, rather than increased circulation longevity of the carrier [7,10]. Unlike drugs such as doxorubicin which form intraliposomal crystals [15] and, consequently, have membrane permeability properties that are independent of lipid composition, vincristine is membrane permeable and its leakage rates can be affected by alterations in lipid composition [7,9,10]. For this reason, vincristine was chosen to determine the effects of lipid composition on the pharmacokinetics, biodistribution and efficacy of a liposomal anticancer drug. It is anticipated that the observations reported here will also apply to other membrane-permeable amphipathic drugs that can be loaded in response to a transmembrane ion gradient [16]. Similar results to those presented here have also been observed in DSPC/cholesterol-based formulations (data not shown). These well-characterized 'conventional' formulations of liposomal vincristine are currently undergoing phase I and phase II clinical testing [17].

The comparisons between liposomes with different PEG-derivatized lipids described here were based on the previously identified [7] increases in vincristine leakage observed for liposomes containing the negatively charged PEG-DSPE (Fig. 1). However, in liposomes containing PEG-DSPE the increased hydrodynamic radius arising from the PEG moiety effectively shields and reduces the negative surface charge to a modest (near-zero) zeta potential [5]. The low electrophoretic mobility of phosphatidylcholine/cholesterol liposomes containing 5 mol% PEG-DSPE (-0.5×10^{-4} cm^2/Vs) reported previously [5] has been confirmed in sphingomyelin/cholesterol liposomes in this study (-0.83×10^{-4} cm^2/Vs; Table 1). In contrast, the conjugation chemistry employed to synthesize the PEG-ceramides results in no net charge (Fig. 1). Therefore, liposomes containing PEG-CER have no surface charge to shield and would be expected to have no detectable electrophoretic mobility (zeta potential or surface charge). This prediction was confirmed in sphingomyelin/cholesterol liposomes containing 5 mol% of PEG-ceramide (Table 1).

Anionic lipids (i.e. phosphatidic acid, phosphatidylserine etc.) that confer a negative surface charge to liposomes [5] also facilitate their rapid removal from the circulation by mediating an increase in protein binding to the liposome surface [6]. Consistent with the observation of low electrophoretic mobilities in SM/chol liposomes containing either PEG-DSPE or PEG-CER (Table 1), the study here shows that these PEG-conjugated lipids conferred very similar pharmacokinetic properties to SM/chol liposomal vincristine (Fig. 3) and DSPC/chol liposomal vincristine (data not shown). It should be noted that a near-zero zeta potential, as assessed by low electrophoretic mobility, does not solely confer slow elimination of liposomal systems from the circulation. This is illustrated by a comparison of SM/chol and SM/chol/PEG-DSPE liposomes, both of which have negligible electrophoretic mobilities (Table 1) but twofold different plasma elimination rates (Fig. 3A). Other factors associated with the PEG-DSPE and PEG-CER formulations, such as comparable protein-binding properties, clearly affect the elimination of these systems from the circulation.

Liposomes prepared with a positive surface charge by the incorporation of a cationic lipid (i.e. stearylamine) have significantly lower permeability to cationic drugs such as verapamil and prochlorperazine [18] as well as vincristine (Boman and Bally, unpublished data) in comparison to neutral liposomes. Therefore, it would be predicted that liposomes bearing a negative surface charge would have increased membrane permeability to vincristine (cationic at intraliposomal pH values) by increasing the local vincristine concentration at the bilayer surface, thereby increasing the transbilayer vincristine concentration gradient. Consistent with this prediction, the anionic

lipids DOPS and DSPG significantly increased the in vitro leakage of vincristine by 90–253% (Table 1). Anionic PEG-DSPE added to SM/chol liposomes also increased the in vivo leakage of vincristine (Fig. 3B) to an extent intermediate (117%) to that effected by DOPS and DSPG (Table 1). In contrast, the neutral PEG-CER was significantly more effective than PEG-DSPE at retaining encapsulated drug and was comparable to neutral SM/chol liposomes both in vitro (Table 1) and in vivo (Fig. 3B). These results suggest that the PEG polymer is able to reduce interactions with the membrane surface, even for small molecules that would be expected to readily gain access to the lipid-water interface.

As part of our efforts to develop drug delivery systems having regulated surface characteristics, we have investigated the potential for PEG-CER to partition out of liposomes at definable rates. This has been achieved by using PEG-CER with different acyl chain lengths and, consequently, differing propensities to partition from the liposome bilayer to the surrounding aqueous medium. Quantification of the exchange of PEG-CER from liposomal bilayers in vitro showed differences in the half-lives in the range between <1 min (C8), 8–10 min (C14) and 22 h (C24) (J. Holland and T.D. Madden, unpublished data). A quantitative relationship between acyl chain length and the rate of interbilayer transfer has also been reported previously for PEG-PE conjugates [19,20]. The in vivo data presented here are consistent with these in vitro results and indicates that SM/chol formulations containing PEG-CER with short acyl chains (C8 and C14) were subject to clearance identical to that observed for SM/chol liposomes lacking PEG-CER (Figs. 4 and 5). We interpret these data to reflect the rapid exchange of short chain PEG-CER out of the SM/chol-based liposomes after i.v. administration and subsequent liposome clearance at rates characteristic of SM/chol liposomes lacking PEG-lipids. Conversely, SM/chol formulations containing PEG-CER with longer acyl chains (i.e. C20 and C24) were not subject to rapid removal in the first 4 h after i.v. administration (Fig. 5A) and displayed plasma liposome concentrations approx. 50% higher than those of SM/chol at 24 h after administration. (Fig. 4). This indicates that the long chain PEG-CER remained in the carriers after administration and conferred increased circulation lifetimes to the liposomes.

A critical element of PEG-lipid conjugates having regulatable partitioning properties is that they do not have a deleterious impact on the retention of encapsulated drugs. Overall, the only formulation with any deleterious effects on vincristine encapsulation and pharmacokinetics was the SM/chol liposome containing the C8 PEG-CER. For liposomal vincristine, the presence of most PEG-CER conjugates did not induce drug leakage, in contrast to PEG-DSPE which increased drug leakage both in vitro (Table 1) and in vivo (Fig. 3B). Similar studies will have to be performed with other encapsulated therapeutic agents to determine if this is behavior is representative of other drug classes.

Taken in sum, these data demonstrate that PEG conjugated to long chain ceramides is very effective at increasing the circulation lifetimes of liposomes. That is, the pharmacokinetics of liposomal carriers containing PEG-lipids is controlled primarily by the hydrophilic PEG moiety, rather than by the hydrophobic domain, and is unaffected by the surface charge conferred by the PEG-to-lipid conjugation chemistry. In contrast, the PEG-ceramides show significantly improved drug retention compared to that observed in similar formulations containing PEG-DSPE. Furthermore, the retention of the PEG-ceramides in the administered liposomes can be manipulated via alterations in the acyl chain length, facilitating the development of regulated release liposomal formulations of therapeutic agents. Finally, the major advantage of the neutral PEG-ceramides arises from the relative independence of the surface-associated PEG off-rates on the surface charge of the lipid carrier. Delivery systems containing nucleic acid-based therapeutics, for example, usually contain cationic lipids to facilitate intracellular delivery. It would be expected that the partition rates of PEG-PE derivatives would be markedly sensitive to the both the cationic lipid content, via electrostatic attraction, and the lipid acyl chain length via hydrophobic interactions. In contrast, the off-rates of the PEG-ceramides would be more readily controlled simply by the strength of the hydrophobic interactions that can be explicitly regulated via the alterations in acyl chain length.

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