Available online at www.sciencedirect.com
ScienceDirect
Biochimica et Biophysica Acta 1768 (2007) 1121–1127
Biochimica et Biophysica Acta BBA
www.elsevier.com/locate/bbamem

Characterization of the drug retention and pharmacokinetic properties of liposomal nanoparticles containing dihydrosphingomyelin

Michael J.W. Johnston [a], Sean C. Semple [b], Sandy K. Klimuk [b], Steve Ansell [b], Norbert Maurer [b], Pieter R. Cullis [a,*]

[a] Department of Biochemistry and Molecular Biology, University of British Columbia, 2146 Health Sciences Mall, Vancouver, BC, Canada V6T 1Z3
[b] Inex Pharmaceuticals Corporation, 100-8900 Glenlyon Parkway, Glenlyon Business Park, Burnaby, BC, Canada V5J 5J8

Received 10 August 2006; received in revised form 4 January 2007; accepted 25 January 2007
Available online 1 February 2007

Abstract

The drug retention and circulation lifetime properties of liposomal nanoparticles (LN) containing dihydrosphingomyelin (DHSM) have been investigated. It is shown that replacement of egg sphingomyelin (ESM) by DHSM in sphingomyelin/cholesterol (Chol) (55/45; mol/mol) LN results in substantially improved drug retention properties both in vitro and in vivo. In the case of liposomal formulations of vincristine, for example, the half-times for drug release ($T_{1/2}$) were approximately 3-fold longer for DHSM/Chol LN as compared to ESM/Chol LN, both in vitro and in vivo. Further increases in $T_{1/2}$ could be achieved by increasing the drug-to-lipid ratio of the liposomal vincristine formulations. In addition, DHSM/Chol LN also exhibit improved circulation lifetimes in vivo as compared to ESM/Chol LN. For example, the half-time for LN clearance ($Tc_{1/2}$) at a low lipid dose (15 μmol lipid/kg, corresponding to 8 mg lipid/kg body weight) in mice was 3.8 h for ESM/Chol LN compared to 6 h for DHSM/Chol LN. In addition, it is also shown that DHSM/Chol LN exhibit much longer half-times for vincristine release as compared to LN with the "Stealth" lipid composition. It is anticipated that DHSM/Chol LN will prove useful as drug delivery vehicles due to their excellent drug retention and circulation lifetime properties.

Keywords: Liposomes; Vincristine; Regulated drug release; Dihydrosphingomyelin; Liposomal pharmacokinetics

1. Introduction

The therapeutic benefits of liposomal nanoparticle (LN) formulations of anticancer drugs include decreased toxicity and increased antitumor efficacy as compared to equivalent doses of the free drug [1–5]. Two important parameters that affect the therapeutic benefits of LN delivery are the circulation lifetimes of the carriers following intravenous (i.v.) injection and the rate of drug release from the liposomes. Long circulation lifetimes lead to enhanced accumulation of LN in disease sites such as tumors [6,7] and slow drug release rates are of particular benefit for cell-cycle specific drugs such as vincristine [8,9]. Extended circulation lifetimes can be achieved using pegylated LN [10], high lipid doses [11] or by using lipids such as sphingomyelin [9]. Many methods have been employed to regulate drug release, including varying the lipid composition of the LN carrier [12], using temperature and/or pH-sensitive lipids [13–15], increasing the drug-to-lipid ratio [16,17] and altering the fatty acyl chain length and saturation of the lipids [18].

In previous work it has been shown that the substitution of egg sphingomyelin for phospholipids such as distearolyphosphatidylcholine (DSPC) in LN results in improved drug retention properties and longer circulation lifetime properties [9]. It has also been shown that LN containing more saturated PC exhibit better drug retention and longer circulation lifetimes

Abbreviations: SM, sphingomyelin; ESM, Egg sphingomyelin; DHSM, dihydrosphingomyelin; BSM, bovine brain sphingomyelin; MSM, bovine milk sphingomyeline; HSPC, Hydrogenated Soybean phosphatidylcholine; DSPE-PEG_{2000}, Distearoyl-*sn*-Glycero-3-Phosphoethanolamine-N-[Poly(ethyleneglycol)2000]; αTC, α-tocopherol; [^{3}H]-CHE, [^{3}H]-cholesterylhexadecyl ether; MLV, multilamellar vesicles; LN, liposomal nanoparticle

* Corresponding author. Department of Biochemistry and Molecular Biology, Life Sciences Center, University of British Columbia, 2350 Health Sciences Mall, Vancouver, BC, Canada V6T 1Z3. Tel.: +1 604 822 4955; fax: +1 604 8224843.
E-mail address: pieterc@interchange.ubc.ca (P.R. Cullis).

0005-2736/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2007.01.019

as compared to LN composed of less saturated PCs [18]. In this work we characterize the behaviour of LN composed of dihydrosphingomyelin (DHSM) in the anticipation that further improvements in drug retention and circulation properties could be achieved employing these more saturated variants of SM. These studies employ vincristine as a representative drug that is relatively "leaky" and difficult to retain in liposomal systems. For example, both doxorubicin and vincristine can be readily accumulated in LN exhibiting a pH gradient, but vincristine leaks out much more rapidly than doxorubicin for LN with the same lipid composition [18]. It is shown that LN containing DHSM exhibit improved vincristine retention and longer circulated lifetimes following intravenous injection as compared to LN composed of ESM.

2. Materials and methods

2.1. Materials

Egg sphingomyelin (ESM), bovine milk sphingomyelin (MSM), hydrogenated soybean phosphatidylcholine (HSPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[poly(ethyleneglycol)2000] (DSPE-PEG_{2000}) were obtained from Northern Lipids Inc.(Vancouver, BC). Bovine brain sphingomyelin (BSM) was purchased from Avanti Polar Lipids (Alabaster, Al, USA). These lipids were used without further purification. Vincristine sulfate was obtained from Fine Chemicals (Cape Town, South Africa). Cholesterol (Chol) was obtained from Sigma (St. Louis, MO, USA) and used without further purification. [^{3}H]-Cholesterylhexadecyl ether (CHE) was obtained from Perkin Elmer Life Sciences (Boston, MA, USA). [^{14}C]-vincristine sulfate was obtained from Chemsyn Laboratories (Lenexa, KA, USA). All other chemicals were obtained from Sigma (St. Louis, MO, USA).

2.2. Synthesis of dihydrosphingomyelin (DHSM)

Twenty-five grams of ESM was dissolved in 250 ml of ethanol and 2.5 g of a 10% palladium/carbon catalyst was added, the lipid and catalyst were then placed under an argon atmosphere. The mixture was heated to 40 °C and stirred for 2 h while hydrogen was passed through the system. Residual hydrogen was removed with an argon flush and any active catalyst was quenched with the addition of cyclohexane. The product was filtered through diatomaceous earth to remove the catalyst, and then subsequently the solvent was removed. Purification of the DHSM was accomplished by dissolving the crude product in ethanol and adding acetone (1:1 v/v) followed by filtration, this precipitation was repeated until the desired purity was obtained. The final product was 99%+ pure based on high performance liquid chromatography (HPLC) with NMR analysis showed no sign of the non-hydrogenated analog.

2.3. Differential scanning calorimetry

Differential scanning calorimetry was carried out on multilamellar vesicles (MLV) composed of the various sphingomyelins, in distilled water, with a Calorimetry Sciences Corporation MC-DSC 4100 instrument (Lindon, UT). Prior to loading into DSC cells, samples were brought to room temperature and degassed for five min under vacuum with stirring. The samples were then vortexed to ensure homogeneity and scanning with a range of 20 to 60 °C at a scan rate of 5 °C/h was immediately carried out.

2.4. Liposome preparation

Lipids and trace amounts of [^{3}H]-CHE were co-dissolved at appropriate molar ratios in ethanol. MLV suspensions were generated after the addition of a 300 mM aqueous solution of magnesium sulfate for liposomes used for drug loading or buffer composed of 20 mM HEPES, 145 mM NaCl pH 7.5 for unloaded liposomes used in pharmacokinetic studies, yielding a final ethanol concentration of 10% (v/v). Liposomal nanoparticles (LN) with diameters of approximately 100 nm were generated by extrusion of MLVs through two stacked Nuclepore polycarbonate filters with a pore size of 100 nm (10 passes) using an extrusion device obtained from Northern Lipids (Vancouver, BC, Canada) [19,20]. For drug loading a transmembrane ion gradient was established through the removal of the external 300 mM magnesium sulfate by dialysis against SEH loading buffer (300 mM sucrose, 3 mM EDTA, 20 mM HEPES, pH 7.4). LN used in comparative pharmacokinetic studies, where no loaded drug was necessary, were dialyzed against 20 mM HEPES, 145 mM NaCl pH 7.5 to remove the remaining ethanol. The mean LN diameter was determined by dynamic light scattering using a NICOMP 370 particle sizer (Nicomp Particle Sizing Inc., Santa Barbara, CA) and found to be 106±36 nm for ESM/Chol LN, 122±51 nm for DHSM/Chol LN and 111±47 nm for the LN composed of HSPC/Chol/DSPE-PEG_{2000}. Phospholipid concentrations were determined using established techniques [21] and the specific activity of the LN was determined using a Beckman LS3801 scintillation counter (Fullerton, CA, USA).

2.5. Vincristine encapsulation in LN

Vincristine was encapsulated into LN using the ionophore-mediated drug loading procedure described previously [22]. Briefly, vincristine sulfate and trace amounts of [^{14}C]-vincristine sulfate were added to LN (5 mM final lipid concentration) at appropriate drug-to-lipid ratios (wt/wt), and subsequently incubated at 65 °C prior to the addition of the calcium ionophore A23187. The LN/drug/ionophore mixture was then incubated at 65 °C for 90 min to provide optimal drug loading conditions. Non-encapsulated vincristine was removed using dialysis against SEH loading buffer [22] prior to quantification of LN-entrapped drug using dual channel counting on a Beckman LS 3801 scintillation counter. Formulations used for in vivo studies were first dialyzed against 300 mM sucrose.

2.6. Assays for in vitro release of vincristine

In vitro drug release assays giving rise to release rates on experimentally convenient timescales were utilized to make quantitative comparisons of drug leakage between vincristine-loaded LN of varying drug-to-lipid ratios. In vitro release assays for liposomal vincristine were conducted using ammonium chloride to degrade the pH gradient [23]. Drug loaded vesicles were diluted with release buffer (2 mM ammonium chloride, 300 mM sucrose, 20 mM HEPES, 3 mM EDTA, pH 7.4) to a lipid concentration of 1.25 mM. The diluted liposomal drug was then placed into dialysis tubing (12–14 K M.Wt. cut off) and dialyzed against release buffer at 50 °C. This temperature was chosen to provide an optimal and convenient in vitro drug leakage rate, generally 50–60% after 60 min for a drug-to-lipid ratio of 0.03(mol/mol). Leakage of vincristine from the loaded LN was assayed by the removal of aliquots for spin column analysis and quantification using dual label liquid scintillation counting [22,24]. The halftimes for drug release ($T_{1/2}$) were calculated from exponential best fits to the release profiles as the time at which the internal drug concentration was half the initial concentration.

2.7. Mice

Female, 6–8 weeks old outbred ICR mice were obtained from Harlan (Indianapolis, IN) and were used for pharmacokinetic and drug payout studies. All mice were quarantined for at least 2 weeks prior to use and kept within a controlled temperature (22±1 °C) and humidity (60±10%) environment. Lighting was maintained on automatic 12 h light/dark cycles. Animal studies were conducted in compliance with the guidelines established by the Canadian Council on Animal Care (CCAC).

2.8. In vivo pharmacokinetics

Vincristine-loaded LN were prepared at various drug-to-lipid (*D*/*L*) ratios and contained 300 mM sucrose as the external buffer. In most instances, drug and lipid concentrations were adjusted to 0.2 mg/ml vincristine and 4 mg/ml total lipid, resulting in doses of 2 mg/kg drug and 40 mg/kg lipid. Empty SM/

Chol liposomes were included in liposomal vincristine formulations prepared with higher *D*/*L* ratios to ensure that mice were injected with an equivalent total lipid dose. Mice were injected via a lateral tail vein and, at appropriate time points, were anesthetized (ketamine/xylazine) and blood was collected via cardiac puncture into EDTA microtainer tubers. Blood was then centrifuged at 400×*g* for 15 min and plasma was collected and de-colorized for lipid and drug determination by dual label liquid scintillation counting as described previously [22].

To assess the pharmacokinetics of three long circulating liposomal formulations (ESM/Chol 55/45 mol%; DHSM/Chol 55/45 mol%; HSPC/Chol/DSPE-PEG_{2000} 57.2/37.6/5.2 mol%), unilamellar liposomes without loaded drug were prepared with an external buffer composed of 20 mM HEPES, 145 mM NaCl pH 7.5. Mice were injected via a lateral tail vein at lipid doses of either 150 μmol/kg or 15 μmol/kg and, at appropriate time points, were anesthetized (ketamine/xylazine overdose) and blood was collected via cardiac puncture into EDTA microtainer tubes. Blood was then centrifuged at 400×*g* for 15 min and plasma was collected and de-colorized for lipid determination by liquid scintillation counting as described previously [22]. Circulation half-times ($Tc_{½}$) were calculated by fitting the plasma LN lipid levels to an exponential function (Sigma Plot 9).

3. Results

3.1. LN containing DHSM exhibit slow drug leakage rates

The first set of experiments was aimed at characterizing the influence of SM species on vincristine release rates from LN. Four species of SM were employed, namely bovine milk SM (MSM), bovine brain SM (BSM), ESM and DHSM. LN composed of SM/Chol (55:45) were made as indicated under Methods and loaded with vincristine to achieve a drug-to-lipid (*D*/*L*) ratio of 0.06 (mol/mol) employing the ionophore loading procedure. In order to achieve vincristine release rates on an experimentally convenient timescale an in vitro release assay consisting of incubation of drug-loaded LN in the presence of 2 mM ammonium chloride at 50 °C was employed. The vincristine release profiles for each liposomal formulation are shown in Fig. 1. As may be noted, LN containing either BSM or MSM release vincristine very rapidly, with halftimes for drug release ($T_{1/2}$) of less than 10 min under the assay conditions employed. LN composed of ESM/Chol exhibit improved retention properties ($T_{1/2}$ ~29 min) whereas DHSM/Chol LN exhibit the slowest drug payout characteristics ($T_{1/2}$ ~61 min). Although similar $T_{1/2}$ values would not be expected in vivo, it would be expected that similar relative values would be observed where the DHSM/Chol LN exhibit the longest retention times. A previous study has shown that vincristine formulations with the longest retention times observed using the ammonium chloride release assay in vitro are also those that exhibit the longest retention times in vivo [17].

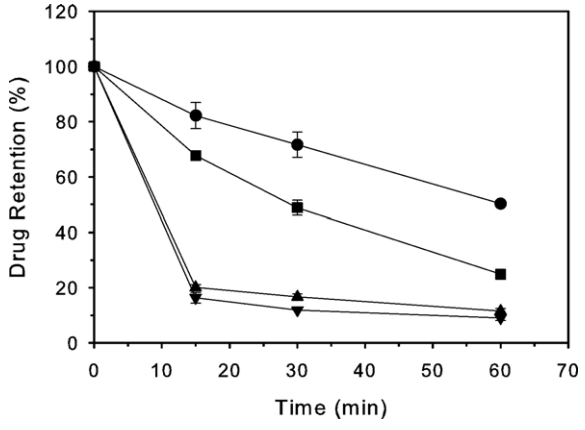

Fig. 1. *In vitro* retention of LN vincristine preparations (0.06, mol/mol) in 2 mM ammonium chloride at 50 °C, composed of various sphingomyelin sources. Bovine milk sphingomyelin (▼), bovine brain sphingomyelin (▲), egg sphingomyelin (■) and dihydrosphingomyelin (●). The lipid concentration in the release assays was 1.25 mM total lipid. Data points represent mean drug retention (±standard deviations) calculated from 3 samples.

Table 1
Acyl chain saturation ratios, phase transition temperatures and half-life for drug release for various sphingomyelin sources

Lipid sample	Acyl chain saturation (%)[a]	Tm, °C	$T_{1/2}$ release, min
Bovine milk sphingomyelin	91	34.2	6.4
Bovine brain sphingomyelin	67	37.8	7.7
Egg sphingomyelin	93	40.8	28.9
Dihydrosphingomyelin	100	46	60.8

[a] As noted from [41].

More unsaturated lipid species usually exhibit lower gel-to-liquid crystalline transition temperatures (Tc) values [25]. The Tc values of the SM species employed here were determined employing differential scanning calorimetry. As shown in Table 1 the transition temperatures vary from 34 °C to 46 °C for the various sphingomyelin species.

3.2. Increasing the drug-to-lipid ratio results in reduced leakage rates in DHSM/Chol LN

The preceding results indicate that vincristine release from DHSM/Chol LN is approximately 2 times slower than from ESM LN. Previous studies have shown that the release rate of vincristine from ESM/Chol LN is also highly sensitive to the *D*/*L* ratio employed, with formulations with higher *D*/*L* values exhibiting much slower drug release rates [16,17]. In order to demonstrate that similar behaviour is also observed for DHSM-containing systems, the in vitro release properties of DHSM/Chol LN loaded to *D*/*L* ratios of 0.027 to 0.158 (mol/mol) were compared. As shown in Fig. 2, the $T_{1/2}$ for vincristine release increased from 35 min to more than 300 min as the *D*/*L* ratio was increased from 0.027 to 0.158 (mol/mol).

3.3. The use of DHSM and high D/L ratios results in additive reductions in vincristine leakage in vivo

The next set of studies was aimed at delineating the in vivo release properties of DHSM/Chol LN loaded with vincristine. As shown in Fig. 3 for LN loaded with vincristine at a *D*/*L* ratio of 0.034 (mol/mol) and administered to outbred ICR mice at a drug dose level of 2 mg/kg, the substitution of DHSM for ESM in SM/Chol LN resulted in an increase in the $T_{1/2}$ for drug release from 8.9 to 16.6 h. Similarly, increasing the *D*/*L* ratio from 0.035 to 0.235 (mol/mol) in the DHSM formulation resulted in an increase in $T_{1/2}$ from 16.6 to 282 h (Fig. 3).

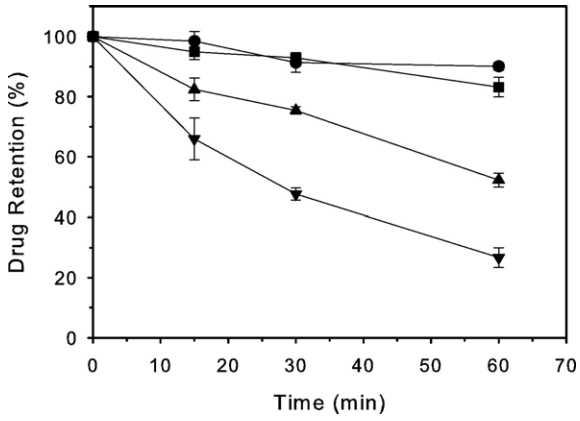

Fig. 2. Vincristine *in vitro* retention for DHSM/Chol LN preparations in 2 mM ammonium chloride at 50 °C as determined for drug to lipid ratios (mol/mol) of 0.027(▼), 0.055 (▲), 0.099 (■) and 0.158 (●). The lipid concentration in the release assays was 1.25 mM total lipid. Data points represent mean drug retention (±standard deviations) calculated from 3 samples.

Because lipid dose can affect the clearance of liposomes from the circulation [11], empty DHSM/Chol liposomes were included with the loaded liposomes formulated at the high *D*/*L* ratio to achieve a constant lipid dose of 40 mg/kg for both formulations.

3.4. DHSM/Chol LN exhibit longer circulation lifetimes than ESM/Chol LN

In addition to drug retention properties, the circulation lifetime of LN following i.v. administration is an important parameter affecting the performance of liposome–drug formulations. This is because long-circulating systems accumulate more effectively at sites of disease such as tumor sites [26], leading to improved therapeutic benefits. LN of various lipid compositions usually exhibit dose dependent pharmacokinetics, with rapid clearance observed at low lipid doses and much slower clearance at higher dose levels [27]. The gold standard for LN with long circulation lifetimes is the so-called Stealth formulation [28] that contains the PEG–lipid DSPE-PEG_{2000}, hydrogenated soy PC (HSPC) and cholesterol and exhibits in vivo blood clearance properties that are relatively dose-independent [29]. The circulation lifetimes of liposomes composed of ESM/Chol (55/45 mol%), DHSM/Chol (55/45 mol%) and the "Stealth" composition (HSPC/Chol/DSPE-PEG_{2000}; 57.2/37.6/5.2 mol%) were therefore examined in female ICR mice. Two lipid doses were employed. The low lipid dose corresponded to 15 μmol phospholipid/kg (8.4 mg lipid/kg for the SM-containing LN; 10.9 mg lipid/kg for the "Stealth" formulation) and the high dose to 150 μmol phospholipid/kg (84 mg lipid/kg for the SM-containing LN;

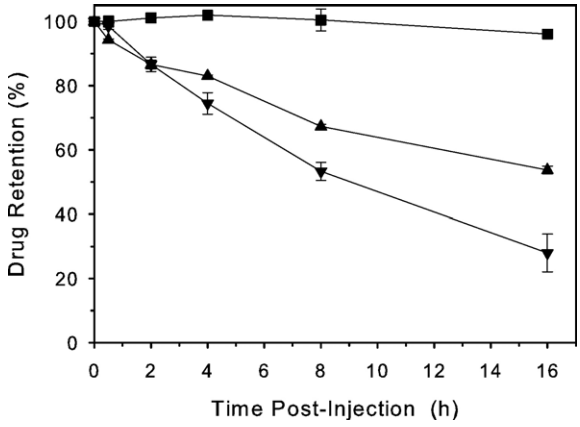

Fig. 3. Vincristine retention in circulating LN composed dihydrosphingomyelin/cholesterol (55/45 mol%) and egg sphingomyelin/cholesterol (55/45 mol%) in mice. Drug-to-lipid ratios (mol/mol) of LN formulations of vincristine composed of DHSM/Chol were 0.034 (▲), 0.235 (■) and ESM/Chol at 0.035 (▼). Mice were dosed at 71 μmol/kg (40 mg/kg) total lipid and 2.4 μmol/kg (2 mg/kg) drug, data points represent mean drug retention (±standard deviations) calculated from 3 mice.

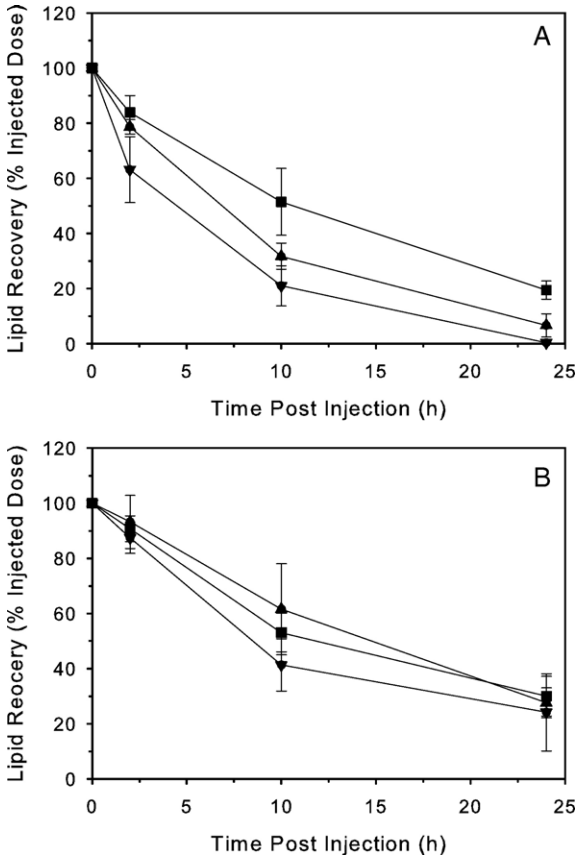

Fig. 4. Lipid recovery in plasma from circulating LN formulated of ESM/Chol (▼) (55/45 mol%), DHSM/Chol (▲) (55/45 mol%) or stealth (■) (HSPC/CH/DSPE-PEG_{2000} 57.2/37.6/5.2 mo%) liposomal formulations. Mice were dosed at either 15 μmol/kg (Panel A) or 150 μmol/kg (Panel B), data points represent mean lipid recovery (±standard deviations) in plasma calculated from 3 mice each.

Table 2
Calculated circulation half-lives ($R^2 \geq 0.98$) for various LN formulations

Liposome composition	$Tc_{1/2}$ 15 μmol/kg	$Tc_{1/2}$ 150 μmol/kg
ESM/Chol (55/45 mol%)	3.8 h	9.4 h
DHSM/Chol (55/45 mol%)	6.0 h	13.2 h
"Stealth" formulation (HSPC/CH/DSPE-PEG_{2000} 57.2/37.6/5.2 mo%)	10.1 h	13 h

109 mg lipid/kg for the "Stealth" formulation). As shown in Fig. 4 and Table 2, the DHSM/Chol LN exhibited significantly longer $Tc_{1/2}$ values than the ESM/Chol LN at both the high lipid doses (13.2 h vs. 9.4 h) and the low lipid doses (6 h vs. 3.8 h). The shorter $Tc_{1/2}$ values observed at the lower lipid doses reflect the dose-dependent clearance behaviour commonly observed for LN formulations [11]. It is interesting to note that the $Tc_{1/2}$ values of the DHSM/Chol LN are equivalent to the "Stealth" formulation at the high dose levels (13.2 h vs. 13 h) and are not markedly shorter even at the low lipid doses (6 h vs. 10.1 h).

3.5. DHSM/Chol LN exhibit significantly improved drug retention properties compared to "Stealth" LN

It is of obvious interest to compare the drug retention properties of "Stealth" LN and DHSM/Chol LN, particularly given the observation that the presence of PEG-containing lipids such as DSPE-PEG_{2000} in LN increases the rate of drug release both in vitro and in vivo [30]. This effect is illustrated in Fig. 5A, where it is shown that the presence of 5.2 mol% DSPE-PEG_{2000} in ESM/Chol LN reduces the half-time for vincristine release from 29 min to 11 min employing the in vitro release assay where the drug release rates have been increased to experimentally convenient time frames by addition of 2 mM NH_4Cl. The relative release rates between DHSM/Chol LN and "Stealth" LN are illustrated in Fig. 5B, from which it may be calculated that DHSM/Chol LN exhibit $T_{1/2}$ values for vincristine release that are approximately 13-fold longer than those achieved by "Stealth" LN.

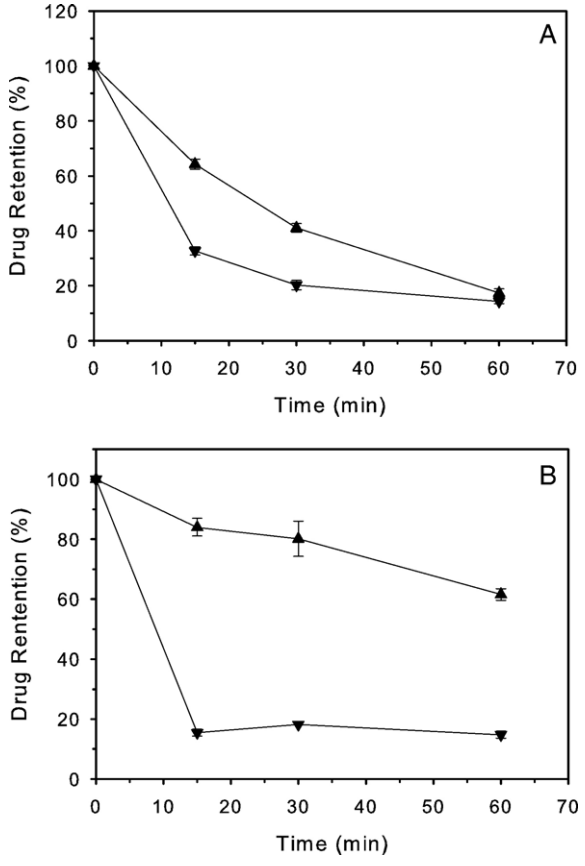

Fig. 5. Comparison of *in vitro* vincristine retention for PEGylated "stealth" and non-PEGylated (ESM/Chol) LN in 2 mM ammonium chloride at 50 °C. Panel A: ESM/Chol (▲) and ESM/Chol containing 5.2 mol% DSPE-$PEG_{(2000)}$ (▼) at *D*/*L* ratios (mol/mol) 0.063 and 0.067 respectively. Panel B: DHSM/Chol (▲) and a PEGylated "stealth" LN formulation (▼) at *D*/*L* ratios (mol/mol) of 0.052 and 0.05 respectively.

4. Discussion

The results of this study indicate that DHSM is a promising lipid for use in liposomal nanoparticle drug delivery applications due to its superior drug retention and circulation lifetime properties. Particular areas of interest concern the reasons why DHSM LN should exhibit such properties, the potential effects of the increased retention on the efficacy of liposomal drug formulations and the biocompatibility of liposomal systems composed of DHSM.

The improved drug retention properties of DHSM as compared to ESM indicate that the DHSM/Chol lipid bilayer provides an improved permeability barrier as compared to that provided by ESM/Chol bilayers. This is consistent with the fact that, as shown here and elsewhere [31], DHSM exhibits a higher gel-to-liquid crystalline transition temperature than ESM, reflecting tighter intermolecular packing of DHSM as compared to ESM. It is also consistent with the finding that DHSM exhibits a greater affinity for cholesterol than ESM [32]. The improvements in drug retention are significant, corresponding to two-fold increases in the half-times for drug release for DHSM/Chol LN loaded with vincristine as compared to ESM/Chol LN. In this context it is interesting that substitution of DSPC for DPPC in PC/Chol LN has little effect on vincristine release properties [33]. DSPC has a transition temperature of 54 °C as compared to 42 °C for DPPC. This large difference in transition temperature may be contrasted to the small difference in transition temperature between DHSM and ESM (~5 °C), for which large gains in drug retention properties are observed. The transition temperatures of component lipids are important because, as demonstrated elsewhere [18], liposomes containing diarachidoynoyl PC (20:0/20:0 PC) or dibehenoyl PC (22:0/22:0 PC), which have transition temperatures of 66 °C and 75 °C respectively, are very difficult to extrude to form LN, and LN formed from these lipids tend to aggregate extensively [18]. This contrasts with the behaviour of the DHSM-containing LN, which are relatively straightforward to extrude and do not exhibit significant aggregation following extrusion.

Previous work has shown that certain drugs such as vincristine [18], vinorelbine [34] and ciprofloxacin [23] are difficult to retain in LN. The importance of achieving slow drug release rates is well established for vincristine, for which it has been shown that the anti-tumor efficacy of a variety of liposomal vincristine formulations with differing release and clearance rates correlates strongly with the circulation half-life of the drug in vivo [35]. This relationship indicates that, for formulations with equivalent circulation lifetimes, the anti-tumor efficacy increases as the half-time for drug release increases. As a result it would be expected that a DHSM/Chol vincristine formulation should exhibit improved efficacy

properties compared to ESM/Chol LN, given that both the carrier circulation lifetime and the half-time for release of the drug are increased. It is also likely that vincristine-containing DHSM/Chol LN will also exhibit improved anti-tumor efficacy as compared to vincristine-containing LN with the "Stealth" lipid composition. This is because although the circulation lifetime may be somewhat shorter for the DHSM/Chol LN, the retention properties of the Stealth LN are markedly less robust as compared to the DHSM/Chol system, potentially leading to substantially shorter drug circulation half-lives.

The improved circulation lifetime properties of DHSM/Chol LN compared to ESM/Chol LN is consistent with the tight packing of the DHSM/Chol lipid bilayer noted above. Previous work has shown that the clearance rate of LN from the circulation is critically dependent on the amount of blood protein adsorbed to the LN surface [36]. The ability of proteins to adsorb onto lipid bilayer membranes is, in turn, dependent on the packing properties of the component lipids (in addition to other factors such as surface charge) [37], where proteins adsorb more readily to bilayers containing more unsaturated lipids. The ability of the PEG coating on "Stealth" LN to extend circulation lifetimes by inhibiting the binding of serum proteins has also been noted [38]. It is also possible that DHSM-containing LN have reduced adsorbed protein levels compared to ESM systems due to reduced surface potentials of DHSM membranes [31].

A final point of discussion concerns the biocompatibility of DHSM/Chol LN, and, in particular, whether DHSM-containing systems could elicit toxic side effects. In this regard it has been shown that DHSM in multilamellar liposomes is readily degraded following i.v. injection [39], although, as indicated by the results presented here, such degradation does not result in undue drug leakage from DHSM/Chol LN over 24 h in vivo. It should also be noted that DHSM is a naturally occurring species of SM. For example, over 50% of the phospholipid associated with the human ocular lens is DHSM [40] and approximately 10% of the SM in cell plasma membranes is DHSM [41]. It is therefore unlikely that DHSM/Chol LN should exhibit any additional toxicity over ESM/Chol LN.

In summary the results of this investigation show that DHSM exhibits improved properties of drug retention and circulation lifetime following i.v. injection as compared to ESM-containing LN. Further, DHSM/Chol LN exhibit superior drug retention properties and comparable circulation lifetime properties as "Stealth" LN. As a result, it is likely that DHSM/Chol LN will be of considerable utility for the in vivo delivery of drugs that are usually hard to retain in liposomal delivery systems.

Acknowledgments

This research was supported by the Canadian Institutes of Health Research and Inex Pharmaceuticals Corporation.

References

[1] J.A. Balazsovits, L.D. Mayer, M.B. Bally, P.R. Cullis, M. McDonell, R.S. Ginsberg, R.E. Falk, Analysis of the effect of liposome encapsulation on the vesicant properties, acute and cardiac toxicities, and antitumor efficacy of doxorubicin, Cancer Chemother. Pharmacol. 23 (1989) 81–86.

[2] Y.K. Oh, D.E. Nix, R.M. Straubinger, Formulation and efficacy of liposome-encapsulated antibiotics for therapy of intracellular *Mycobacterium avium* infection, Antimicrob. Agents Chemother. 39 (1995) 2104–2111.

[3] H. Lim, D. Masin, T. Madden, M. Bally, Influence of drug release characteristics on the therapeutic activity of liposomal mitoxantrone, J. Pharmacol. Exp. Ther. 281 (1997) 566–573.

[4] M. Grunaug, J.R. Bogner, O. Loch, F.D. Goebel, Liposomal doxorubicin in pulmonary Kaposi's sarcoma: improved survival as compared to patients without liposomal doxorubicin, Eur. J. Med. Res. 3 (1998) 13–19.

[5] C. Leonetti, M. Scarsella, S.C. Semple, A. Molinari, C. D'Angelo, A. Stoppacciaro, A. Biroccio, G. Zupi, In vivo administration of liposomal vincristine sensitizes drug-resistant human solid tumors, Int. J. Cancer 110 (2004) 767–774.

[6] D.C. Drummond, O. Meyer, K. Hong, D.B. Kirpotin, D. Papahadjopoulos, Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors, Pharmacol. Rev. 51 (1999) 691–743.

[7] F. Yuan, M. Dellian, D. Fukumura, M. Leunig, D.A. Berk, V.P. Torchilin, R.K. Jain, Vascular permeability in a human tumor xenograft: molecular size dependence and cutoff size, Cancer Res. 55 (1995) 3752–3756.

[8] L. Mayer, R. Nayar, R. Thies, N. Boman, P. Cullis, M. Bally, Identification of vesicle properties that enhance the antitumour activity of liposomal vincristine against murine L1210 leukemia, Cancer Chemother. Pharmacol. 33 (1993) 17–24.

[9] M.S. Webb, T.O. Harasym, D. Masin, M.B. Bally, L.D. Mayer, Sphingomyelin-cholesterol liposomes significantly enhance the pharmacokinetic and therapeutic properties of vincristine in murine and human tumour models, Br. J. Cancer 72 (1995) 896–904.

[10] T.M. Allen, C. Hansen, F. Martin, C. Redemann, A. Yau-Young, Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo, Biochim. Biophys. Acta 1066 (1991) 29–36.

[11] C.D. Oja, S.C. Semple, A. Chonn, P.R. Cullis, Influence of dose on liposome clearance: critical role of blood proteins, Biochim. Biophys. Acta 1281 (1996) 31–37.

[12] L.D. Mayer, L.C. Tai, D.S. Ko, D. Masin, R.S. Ginsberg, P.R. Cullis, M.B. Bally, Influence of vesicle size, lipid composition, and drug-to-lipid ratio on the biological activity of liposomal doxorubicin in mice, Cancer Res. 49 (1989) 5922–5930.

[13] T. Tomita, M. Watanabe, T. Takahashi, K. Kumai, T. Tadakuma, T. Yasuda, Temperature-sensitive release of adriamycin, an amphiphilic antitumor agent, from dipalmitoylphosphatidylcholine-cholesterol liposomes, Biochim. Biophys. Acta 978 (1989) 185–190.

[14] S. Ning, K. Macleod, R.M. Abra, A.H. Huang, G.M. Hahn, Hyperthermia induces doxorubicin release from long-circulating liposomes and enhances their anti-tumor efficacy, Int. J. Radiat. Oncol. Biol. Phys. 29 (1994) 827–834.

[15] S. Zellmer, G. Cevc, Tumor targeting in vivo by means of thermolabile fusogenic liposomes, J. Drug Target 4 (1996) 19–29.

[16] S.A. Abraham, K. Edwards, G. Karlsson, N. Hudon, L.D. Mayer, M.B. Bally, An evaluation of transmembrane ion gradient-mediated encapsulation of topotecan within liposomes, J. Control Release 96 (2004) 449–461.

[17] M.J. Johnston, S.C. Semple, S.K. Klimuk, K. Edwards, M.L. Eisenhardt, E.C. Leng, G. Karlsson, D. Yanko, P.R. Cullis, Therapeutically optimized rates of drug release can be achieved by varying the drug-to-lipid ratio in liposomal vincristine formulations, Biochim. Biophys. Acta 1758 (2006) 55–64.

[18] N.L. Boman, L.D. Mayer, P.R. Cullis, Optimization of the retention properties of vincristine in liposomal systems, Biochim. Biophys. Acta 1152 (1993) 253–258.

[19] L.D. Mayer, M.J. Hope, P.R. Cullis, Vesicles of variable sizes produced by a rapid extrusion procedure, Biochim. Biophys. Acta 858 (1986) 161–168.

[20] M.J. Hope, M.B. Bally, G. Webb, P.R. Cullis, Production of large unilamellar vesicles by a rapid extrusion procedure: characterization of size, trapped volume and ability to maintain a membrane potential, Biochim. Biophys. Acta 812 (1985) 55–65.

[21] C. Fiske, Y. Subbarow, The colorimetric determination of phosphorus, J. Biol. Chem. 66 (1925) 375–400.
[22] D.B. Fenske, K.F. Wong, E. Maurer, N. Maurer, J.M. Leenhouts, N. Boman, L. Amankwa, P.R. Cullis, Ionophore-mediated uptake of ciprofloxacin and vincristine into large unilamellar vesicles exhibiting transmembrane ion gradients, Biochim. Biophys. Acta 1414 (1998) 188–204.
[23] N. Maurer, K.F. Wong, M.J. Hope, P.R. Cullis, Anomalous solubility behavior of the antibiotic ciprofloxacin encapsulated in liposomes: a 1H-NMR study, Biochim. Biophys. Acta 1374 (1998) 9–20.
[24] L.D. Mayer, M.B. Bally, H. Loughrey, D. Masin, P.R. Cullis, Liposomal vincristine preparations which exhibit decreased drug toxicity and increased activity against murine L1210 and P388 tumors, Cancer Res. 50 (1990) 575–579.
[25] P.R. Cullis, D.B. Fenske, M.J. Hope, in: D.E. Vance, J.E. Vance (Eds.), Biochemistry of Lipids and Membranes, Elsevier, Amsterdam, 1996, pp. 1–34.
[26] A.A. Gabizon, Selective tumor localization and improved therapeutic index of anthracyclines encapsulated in long-circulating liposomes, Cancer Res. 52 (1992) 891–896.
[27] S. Semple, A. Chonn, P. Cullis, Interactions of liposomes and lipid-based carriers with blood proteins: relation to clearance behavior in vivo, Adv. Drug Delivery Rev. 32 (1998) 3–17.
[28] L. Cattel, M. Ceruti, F. Dosio, From conventional to stealth liposomes: a new frontier in cancer chemotherapy, Tumori 89 (2003) 237–249.
[29] T.M. Allen, C. Hansen, Pharmacokinetics of stealth versus conventional liposomes: effect of dose, Biochim. Biophys. Acta 1068 (1991) 133–141.
[30] M.S. Webb, D. Saxon, F.M.P. Wong, H.J. Lim, Z. Wang, M.B. Bally, L.S.L. Choi, P.R. Cullis, L.D. Mayer, Comparison of different hydrophobic anchors conjugated to poly(ethylene glycol): effects on the pharmacokinetics of liposomal vincristine, Biochim. Biophys Acta, Biomembr. 1372 (1998) 272–282.
[31] M. Kuikka, B. Ramstedt, H. Ohvo-Rekila, J. Tuuf, J.P. Slotte, Membrane properties of D-*erythro*-N-acyl sphingomyelins and their corresponding dihydro species, Biophys. J. 80 (2001) 2327–2337.
[32] L.K. Li, L. So, A. Spector, Membrane cholesterol and phospholipid in consecutive concentric sections of human lenses, J. Lipid Res. 26 (1985) 600–609.
[33] N. Boman, Thesis—Department of Biochemistry, pp. 120, University of British Columbia, Vancouver 1994.
[34] S.C. Semple, R. Leone, J. Wang, E.C. Leng, S.K. Klimuk, M.L. Eisenhardt, Z.N. Yuan, K. Edwards, N. Maurer, M.J. Hope, P.R. Cullis, Q.F. Ahkong, Optimization and characterization of a sphingomyelin/cholesterol liposome formulation of vinorelbine with promising antitumor activity, J. Pharm. Sci. 94 (2005) 1024–1038.
[35] N.L. Boman, P.R. Cullis, L.D. Mayer, M.B. Bally, M.S. Webb, in: M.C. Woodle, G. Storm (Eds.), Long Circulating Liposomes: Old Drugs, New Therapeutics, Landes Bioscience, Austin, TX, 1998, pp. 29–49.
[36] A. Chonn, S.C. Semple, P.R. Cullis, Association of blood proteins with large unilamellar liposomes in vivo. Relation to circulation lifetimes, J. Biol. Chem. 267 (1992) 18759–18765.
[37] S. Semple, A. Chonn, P. Cullis, Influence of cholesterol on the association of plasma proteins with liposomes, Biochemistry 35 (1996) 2521–2525.
[38] J. Senior, C. Delgado, D. Fisher, C. Tilcock, G. Gregoriadis, Influence of surface hydrophilicity of liposomes on their interaction with plasma protein and their clearance from the circulation: studies with poly(ethylene glycol)-coated vesicles, Biochim. Biophys. Acta 1062 (1991) 77–82.
[39] P.B. Schneider, E.P. Kennedy, Metabolism of labeled dihydrosphingomyelin in vivo, J. Lipid Res. 9 (1968) 58–64.
[40] W.C. Byrdwell, D. Borchman, R.A. Porter, K.G. Taylor, M.C. Yappert, Separation and characterization of the unknown phospholipid in human lens membranes, Invest. Ophthalmol. Visual Sci. 35 (1994) 4333–4343.
[41] B. Ramstedt, P. Leppimaki, M. Axberg, J.P. Slotte, Analysis of natural and synthetic sphingomyelins using high-performance thin-layer chromatography, Eur. J. Biochem. 266 (1999) 997–1002.