A Cationic Liposomal Vincristine Formulation with Improved Vincristine Retention, Extended Circulation Lifetime and Increased Anti-Tumor Activity

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Abstract: A critical characteristic of optimized liposomal delivery systems is controlled drug release. This report describes the use of the monoacyl cationic lipids stearylamine (SA) and sphingosine (SPH), in conjunction with a transmembrane pH gradient, to enhance retention of the anticancer agent vincristine within liposomes administered intravenously to mice. The addition of SA to liposomes composed of distearoylphosphatidylcholine (DSPC) and cholesterol (Chol), to achieve a final composition of DSPC/Chol/SA at ratios of 45/45/10 (mol/mol/mol), caused a significant improvement in vincristine retention in the circulation of mice when combined with a transmembrane pH gradient of 5 units. Specifically, the presence of SA in liposomes having an internal pH (pH_i) of 2.0 had a plasma vincristine AUC of 41.8 mg•h/100 mL plasma, compared to a vincristine AUC in the plasma of 28.0 mg•h/100 mL for identical liposomes lacking SA; an increase of 49%. Similar effects were observed with 10 mol% of the cationic lipid SPH. Comparison to control formulations lacking SA or SPH, or with smaller pH gradients (3 units), showed that improved vincristine retention required both the cationic lipid and the larger pH gradient. The presence of SA or SPH significantly improved vincristine pharmacokinetics due to the increased drug retention, but did not adversely alter the liposome pharmacokinetics. Enhanced retention of vincristine using SPH and a transmembrane pH gradient was associated with significant increases in anti-tumor activity against the murine P388 leukemia. The results are directly relevant to the use of charged lipid components to improve the drug retention/release attributes and the use of cationic lipids to promote accumulation at tumor-associated vascular endothelium.

Keywords: Liposome, Vincristine, Cationic lipid, Pharmacokinetics, Cancer.

INTRODUCTION

A wide variety of clinically effective drugs are characterized as having a narrow "therapeutic window", a small range between the dose at which therapeutic activity occurs and the dose at which deleterious side effects limit the use of higher drug doses. As the utility of such agents would be increased with a broader therapeutic window, attempts to optimize this parameter in the clinical setting have included supportive medications, dose and schedule changes, extended-duration infusions and the use of drug delivery systems [1, 2].

Liposomal drug delivery systems have been tested extensively for their potential to increase the effectiveness, and reduce the toxicity, of a number of drugs. A critical performance characteristic of an optimized liposomal drug formulation is achieving a drug release rate appropriate for treatment of the disease or condition [2-7]. For example, in previous studies of liposomal formulations of the anticancer drug vincristine, liposome compositions having enhanced drug retention characteristics were associated with increased plasma vincristine concentrations and significantly improved antitumor activity [5, 7]. The central role of drug retention in defining the performance of liposomal drug formulations has been reviewed in detail [6]. In a separate study [8], we described a liposomal formulation that was designed to increase the retention of cationic drugs within the liposomes by using the cationic lipid stearylamine. Specifically, DSPC/Chol liposomes containing 10 mol% stearylamine were loaded with the cationic drugs using a transbilayer pH-gradient driving force [8]. In the presence of a transbilayer pH gradient, the stearylamine rapidly and completely redistributed to the inner monolayer of the liposome [9, 10]. This redistribution of cationic lipid to the inner monolayer of the liposomal membrane conferred a significant positive

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surface charge density that reduced the *in vitro* leakage of the encapsulated cationic drugs verapamil and prochlorperazine.

The ability of cationic lipids to decrease the release rate of cationic drugs from liposomes could be considered of only academic interest given evidence that charged lipids enhance liposome elimination following i.v. administration [11]. That is, improvements in drug retention achieved with cationic lipids would be offset by increased liposome elimination rates. More recently, however, there is a renewed interest in the development of charged liposomal drug delivery systems. This interest has emerged for two reasons. First, strategies have been defined through which cationic or anionic liposomes can be designed to have circulation lifetimes comparable to neutral formulations. Specifically, this can be achieved through use of PEG-modified lipids incorporated at appropriate levels within the liposomes [12, 13] or, as described here, by using a transmembrane ion gradient to engender transmembrane flip-flop of a charged, monoacyl, lipid to the inner leaflet of the liposomal membrane [8-10]. Second, and perhaps of greatest interest, charged lipids have the potential to improve therapeutic effects of associated drugs. For example, investigators have recently pursued the development of cationic liposomes based on evidence that the cationic lipid can enhance vascular accumulation at tumor sites. A recent study by Schmitt-Sody and colleagues [14] suggested that cationic liposomes achieve selective and efficient drug delivery to tumor-associated microvessels. This work focused on a novel liposomal formulation of paclitaxel which exhibited significant therapeutic activity in a melanoma tumor model. In addition, Campbell and colleagues [15] demonstrated vascular accumulation of cationic liposomes in LS174T tumors. These authors speculated that irregular tumor blood flow may enhance cationic liposome interactions with anionic sites specifically expressed on tumor-associated blood vessels. Thurston and colleagues [16] reported that this association may be diagnostically and clinically useful, based on the idea that cationic liposomes could selectively target angiogenic blood vessels. This study highlighted results showing that angiogenic endothelial cells averaged 15-33 fold more uptake (of cationic liposomes) than corresponding normal endothelial cells.

The studies reported here represent an extension of the earlier *in vitro* work [8] to the *in vivo* setting and validates the utility of this approach in animals. Specifically, this report demonstrate that improved drug retention, enhanced circulation longevity and improved therapeutic effects in an *in vivo* setting can be achieved by using cationic lipids. To investigate these issues, the anticancer agent vincristine was encapsulated into liposomes comprised of DSPC and cholesterol, with or without the cationic lipid stearylamine or sphingosine. Liposomes based on DSPC and cholesterol were used for the encapsulation of vincristine due to our extensive previous *in vivo* experience with this liposome and drug combination [5, 6 and references cited therein]. The results show that the incorporation of these cationic lipids increases vincristine retention within DSPC/cholesterol liposomes. The presence of these weakly basic lipids has no effect on liposome circulation time *in vivo*, but results in a dramatic improvement in the pharmacological activity of the formulation.

MATERIALS AND METHODS

Materials

DSPC was obtained from Avanti Polar Lipids. Cholesterol (Chol), stearylamine (SA), and sphingosine (SPH) were purchased from Sigma Chemical Company (St. Louis, MO). Vincristine sulfate was purchased from the BC Cancer Agency Pharmacy. [^{14}C]-cholesteryl hexadecyl ether, custom synthesized by Amersham (Oakville, Ontario), was chosen as a lipid marker since it is not exchanged or metabolized *in vivo* [17]. [^{3}H]-vincristine was purchased from Amersham. Female BDF1 mice (18-22 g) were obtained from Charles River Laboratories (Quebec, Canada). P388 murine lymphocytic leukemia cells were obtained from the tumor repository of the Developmental Therapeutic Program of the National Cancer Institute.

Preparation of liposomes

DSPC/Chol (55/45; mol/mol), DSPC/Chol/SA (45/45/10; mol/mol/mol), or DSPC/Chol/SPH (45/45/10; mol/mol/mol) solutions were prepared by dissolving the lipid mixtures in 95% ethanol (1 mL/100 mg total lipid). These mixtures were heated at 60°C for 30 minutes then multilamellar vesicles were formed by the addition of a preheated (60°C) solution of 300 mM citric acid (pH 4.0 or pH 2.0) to a final concentration of 25 mg lipid/mL. These samples were heated at 60°C for an additional 30 minutes then the multilamellar vesicles were extruded 10 times through two polycarbonate filters having pores with a diameter of 100 nm [18, 19] to produce large unilamellar liposomes with mean diameters between 100 and 140 nm based on quasi-elastic light scattering (NICOMP Model 270 sub-micron particle sizer). The extrusion device was obtained from Northern Lipids, Inc. (Vancouver, B.C.) and was operated at 60°C. Ethanol was subsequently removed from the liposomes by dialysis (Spectra/Por 2 dialysis tubing, 12,000-14,000 MWCO) for 24 h against 2 changes of 10 volumes of 300 mM citric acid (pH 4.0 or pH 2.0). Studies by other investigators have shown that liposomes composed of DSPC and cholesterol are physically and chemically stable during exposure to acidic (pH 2.0) solutions, even at 37°C [20-22]. Vincristine sulfate, supplied as a solution with a pH as low as 3.5, is more susceptible to high pH values (> 10) than low pH values (degradation requires 3 M sulphuric acid) was stable during the course of these studies.

Vincristine Encapsulation into Liposomes

Vincristine was encapsulated in liposomes using the pH-gradient based loading procedure described elsewhere [23]. Liposome preparations were passed over a G-25 Sephadex gel filtration column that had been pre-equilibrated with HBS (pH 7.4) to achieve a pH-gradient across the vesicle membranes. Vincristine (vincristine sulfate solution, 1 mg vincristine/mL) was added to the liposome suspension to achieve a drug/lipid ratio of 0.1/1.0 (wt/wt). The resulting exterior pH of the liposome/vincristine mixture was raised to pH 7.4 with 0.5 M Na_2HPO_4 and immediately heated to 60°C for 10 minutes. This procedure ensured > 95% trapping efficiencies in all cases.

Animal Studies

All animal studies outlined below were completed using protocols approved by the institutional (University of British Columbia) animal care committee, and met the guidelines of the Canadian Council of Animal Care. Pharmacokinetic studies were performed by injecting empty liposomes or liposomal vincristine preparations into BDF1 mice *via* a lateral tail vein at a dose of 2 mg vincristine/kg (20 mg lipid/kg). It has been shown previously [23] that this dose of liposomal vincristine exhibits measurable levels of antitumor activity in the murine P388 leukemia model. At various time points, mice were anaesthetized with i.p. ketamine (160 mg/kg) and xylazine (20 mg/kg) and blood removed *via* cardiac puncture and collected in EDTA-coated microtainer tubes (Becton Dickenson). Plasma was obtained by centrifugation (500 x g for 10 minutes) and liposomal lipid and/or vincristine quantities were determined using dual label scintillation counting (Packard 1900TR). Area-under-the-curve (AUC) values were calculated from the vincristine or lipid clearance curves (mg/100 mL plasma vs. time) using the trapezoidal algorithm for unequally spaced x values (SigmaPlot 2002 for Windows Version 8.0, SPSS Inc.).

Biodistribution studies were performed on the same mice used for pharmacokinetic studies. Following cardiac puncture, mice were terminated by cervical dislocation, and the liver and spleen were removed from each animal and weighed. Tissue homogenates in saline (10% w/v) were prepared using a Polytron homogenizer (Brinkmann Instruments, Rexdale, Ontario) then 500 mL of each homogenate was digested with 500 mL of "Solvable" (DuPont Canada, Inc., Mississauga, Ontario) for 1 h at 50°C. After cooling to room temperature, the samples were decolorized with 200 mL of 30% hydrogen peroxide and maintained at 4°C overnight to prevent excessive foaming. Samples were assayed by liquid scintillation counting as described above.

The anti-tumor properties of liposomal vincristine formulations were evaluated against the P388 lymphocytic leukemia model. BDF1 mice (5 per group) were injected intraperitoneally with 1 x 10^5 P388 cells. The indicated doses of saline, free vincristine or liposomal vincristine were

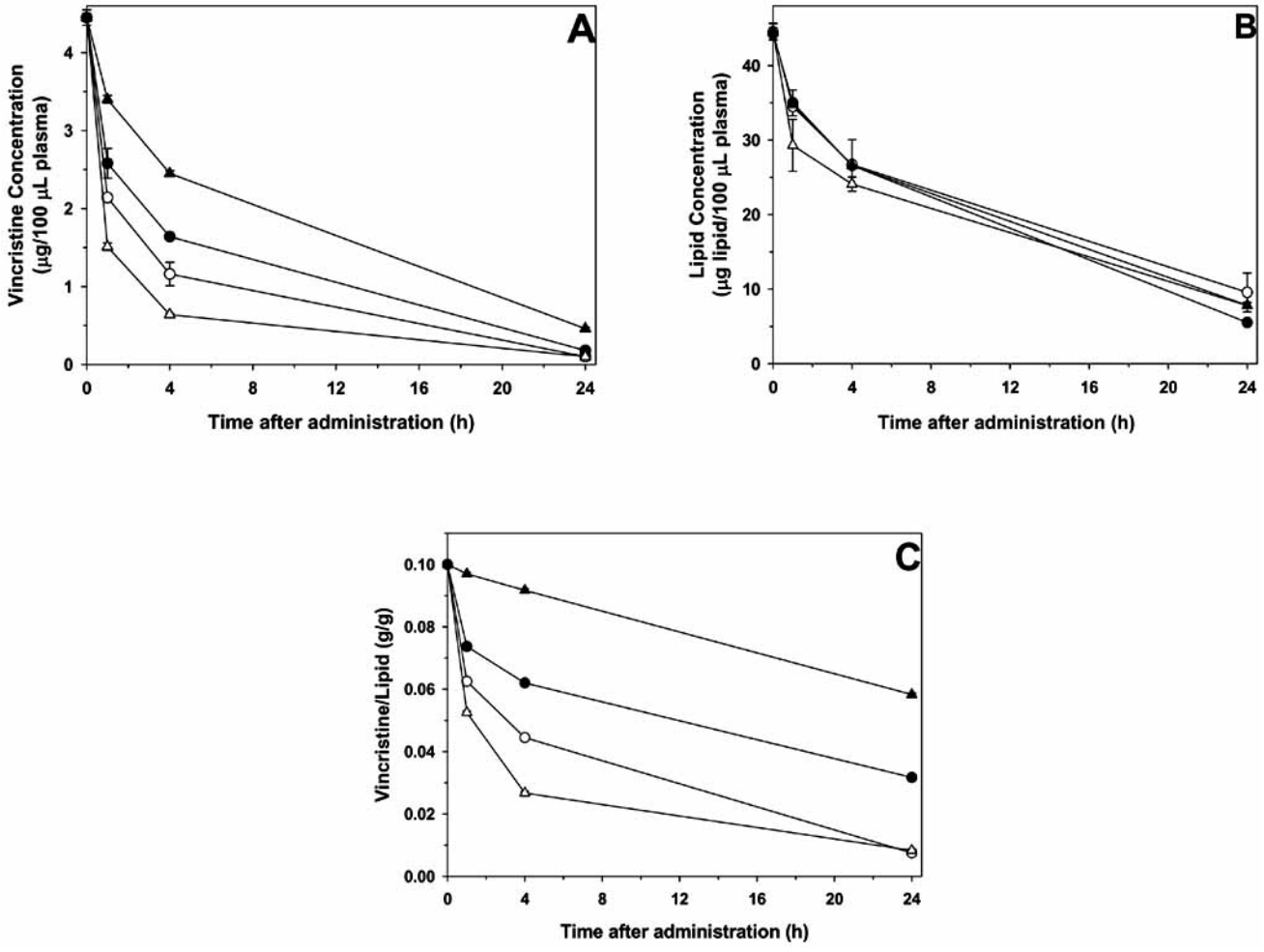

Fig. (1). Influence of stearylamine and internal pH on lipid and drug clearance *in vivo*. Plasma concentrations of vincristine (**A**) and liposomal lipid (**B**) as well as vincristine/lipid ratios (**C**) were determined following i.v. administration in BDF1 mice of DSPC/Chol pH_i 4.0 (○), DSPC/Chol pH_i 2.0 (●), DSPC/Chol/SA pH_i 4.0 (D) and DSPC/Chol/SA pH_i 2.0 (s). Vincristine was encapsulated at a drug/lipid ratio of 0.1/1.0 (wt/wt). Error bars represent the mean and standard deviations from four mice.

given by intravenous administration 24 h after the tumor inoculation. Animal weights and mortality were monitored daily. Median survival times and p-values were determined using the nonparametric Mann-Whitney-Wilcoxon Rank Sum procedure.

RESULTS AND DISCUSSION

Plasma Clearance Studies

Previous work has shown that the presence of a transmembrane pH-gradient across a liposomal membrane (acidic inside) induces the complete migration of cationic lipids such as stearylamine and sphingosine to the inner monolayer [9, 10]. It has also been shown previously that the presence of 10 mol% stearlyamine in a DSPC/Chol liposomal formulation significantly improves the retention of cationic drugs (verapamil and prochlorperazine) when the internal pH is low (2.0) [8]. To determine if these effects could be observed *in vivo*, DSPC/Chol liposomes were prepared with or without 10% stearylamine and vincristine was encapsulated in these formulations using a transmembrane pH gradient of approximately 3.0 (pH_i 4.0) or 5.0 (pH_i 2.0) units. After i.v. administration, there was a significant increase of vincristine plasma concentrations *in vivo* in those formulations having an internal pH of 2.0 (Fig. **1A**); an effect which was not observed in DSPC/Chol/SA formulations loaded using an internal pH of 4.0. This is most apparent by examination of the calculated area-under-the-curve (AUC) values for plasma vincristine: 41.8 mg•h/100 mL plasma for DSPC/Chol/SA (pH_i 2.0), 28.0 mg•h/100 mL plasma for DSPC/Chol (pH_i 2.0), 20.8 mg•h/100 mL plasma for DSPC/Chol (pH_i 4.0) and 13.6 mg•h/100 mL plasma for the DSPC/Chol/SA (pH_i 4.0) formulation. That is, the presence of SA in liposomes having in internal pH of 2.0 increased the plasma vincristine AUC by 49% compared to identical liposomes lacking SA.

Importantly, changes of lipid composition or intraliposomal pH had no effect on liposome elimination rates from the plasma (Fig. **1B**). The calculated area-under-the-curve (AUC) values for the liposomal lipid in the plasma were very similar: 475.9, 453.3, 493.9 and 436.0 mg•h/100 mL plasma for the DSPC/Chol/SA (pH_i 2.0), DSPC/Chol (pH_i 2.0), DSPC/Chol (pH_i 4.0) and DSPC/Chol/SA (pH_i 4.0) formulations, respectively. For this reason it can be concluded that higher plasma concentrations of vincristine (Fig. **1A**) in DSPC/Chol (pH_i 2.0) and DSPC/Chol/SA (pH_i 2.0) formulations were due to a significant increase of vincristine retention within these liposomes, a result that is confirmed in Fig. (**1C**) which shows the vincristine/liposomal lipid ratios in the plasma following i.v. administration. At 24 h after administration, there was approximately a 6-fold higher vincristine/lipid ratio for the stearylamine-containing liposomes at pH_i 2.0 than for the DSPC/Chol (pH_i 4.0) liposomes and a 2-fold increase of vincristine retention compared to the DSPC/Chol (pH_i 2.0) vesicles. The highest plasma concentrations of vincristine were observed in the DSPC/Chol SA (pH_i 2.0) liposomes (Fig. **1A**), a result of the superior retention of drug in this formulation (Fig. **1C**). Overall, these data strongly indicate that the incorporation of the cationic lipid stearylamine increases vincristine retention within DSPC/Chol-based liposomes *in vivo*.

This improved retention of vincristine was attributable primarily to the presence of stearylamine, rather than the

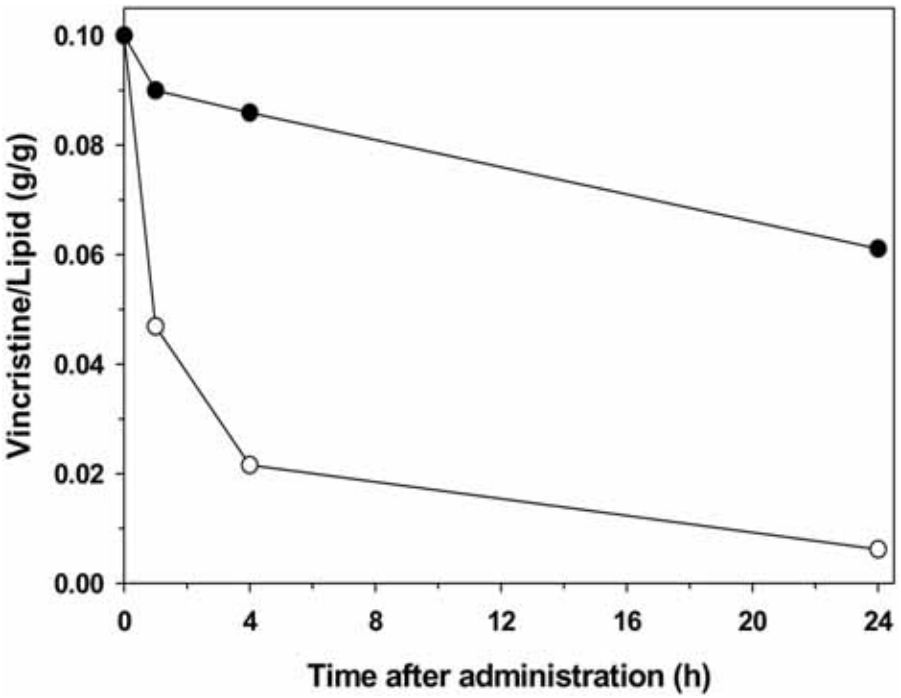

Fig. (2). Effects of sphingosine (SPH) on vincristine retention in liposomes after intravenous administration. Vincristine/lipid ratios were determined with the incorporation of 10 mol% SPH in DSPC/Chol based liposomes. Vincristine retention was determined following i.v. administration in BDF1 mice for liposomes prepared having pH_i 4.0 (○) or pH_i 2.0 (●). Vincristine was encapsulated at a drug/lipid ratio of 0.1/1.0 (wt/wt).

intraliposomal pH alone, as the control formulation of DSPC/Chol (pH_i 2.0) had more rapid vincristine leakage, lower plasma vincristine concentrations and was similar to the DSPC/Chol (pH_i 4.0) formulation (Fig. **1A** and **1C**). It should be noted that the improved vincristine retention at pH_i of 2.0, but not at pH_i of 4.0 (Fig. **1**), was expected based on our previous *in vitro* results and mathematical modeling [8]. Specifically, in our model of stearylamine activity, which is based on the development of a positive surface charge density on the inner monolayer of the liposome [8], an influence of stearylamine would not be expected at a pH_i of 4.0 due to the presence of significant quantities of negatively charged citrate (lowest pK = 3.1) in the intraliposomal compartment. This anionic citrate effectively neutralizes the cationic stearylamine located on the inner monolayer at pH_i 4.0, preventing the development of a positive surface charge density and precluding the lipids ability to affect drug leakage. The model predicted that an intraliposomal pH lower than the lowest citrate pK value of 3.1 (i.e., pH_i 2.0) would be necessary to neutralize all anionic citrate, allow the development of a positive surface charge on the internal monolayer and prevent cationic drug leakage, as was observed both *in vitro* [8] and *in vivo* (Fig. **1**).

Since sphingosine (SPH) is a naturally occurring lipid in biological membranes [24-26], and concerns about stearylamine-related toxicities exist [27, 28], we determined if SPH also facilitated the retention of vincristine in DSPC/Chol based liposomes. Vincristine retention profiles, as illustrated by vincristine/lipid ratios following i.v. administration (Fig. **2**), suggest that formulations having 10 mol% of SPH had drug retention characteristics almost identical to those obtained using the stearylamine-containing formulation (see Fig. **1C**). In both liposomal formulations, the presence of a cationic lipid substantially improved drug retention at pH_i 2.0, but not at pH_i 4.0. It should be noted that liposome elimination rates in the SPH-containing formulations were essentially identical to those formulations prepared without SPH (results not shown).

As indicated in the Introduction, previous studies suggest that the presence of cationic lipids in a liposomal formulation decrease the circulation times of the liposomes [11]. This is clearly not the case for the SA- and SPH-containing formulations described here. This is likely due to the fact that the pH gradient across the membrane facilitates transbilayer flip-flop of the cationic lipid to the inner leaflet of the liposomal membrane. This suggestion is consistent with the data presented in Fig. (**1B**) showing that the clearance rate of the cationic liposomal formulations of vincristine is identical to that of formulations lacking cationic lipid. To demonstrate this more directly, the plasma elimination of SPH-containing liposomes was determined in the absence of encapsulated drug and the absence of a transmembrane pH gradient. DSPC/Chol/SPH (45/45/10; mol/mol/mol) liposomes were prepared with an internal buffer system of HBS at pH 7.5 or Citrate buffer at pH 4.0 and subsequently injected i.v. into BDF1 mice. The results show that SPH liposomes having both interior and exterior pH of 7.5 (i.e., no transmembrane pH gradient) had the most rapid elimination from the blood compartment (Fig. **3**). In contrast, the DSPC/Chol/SPH formulation possessing a transmembrane pH gradient (pH_i 4.0) had a reduced plasma elimination rate that was very similar to that obtained for DSPC/Chol liposomes lacking SPH. The data shown in (Fig. **3**) are confirmed by comparison of the calculated area-under-the-curve (AUC) values for the liposomal lipid in the plasma: 287.5, 256.5 and 166.4 mg•h/100 mL plasma for the DSPC/Chol (pH_i 7.5), DSPC/Chol/SPH (pH_i 4.0) and DSPC/Chol/SPH (pH_i 7.5) formulations, respectively. It is notable that the SPH formulation with the cationic lipid

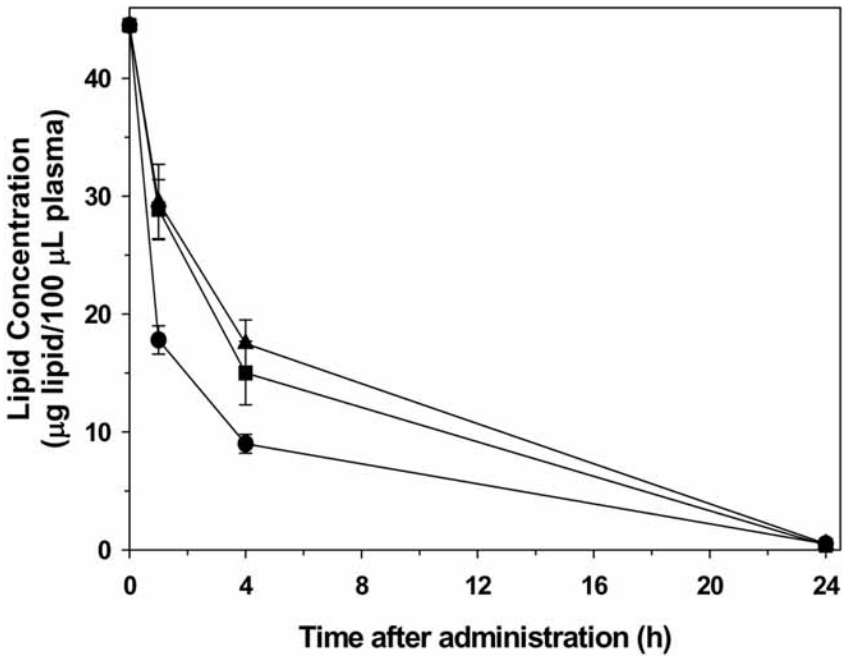

Fig. (3). The effect of a pH-gradient on the clearance of DSPC/Chol/SPH liposomes *in vivo*. Liposome clearance was determined following i.v. administration in BDF1 mice of empty DSPC/Chol/SPH (pH_i 7.5) (●), DSPC/Chol/SPH (pH_i 4.0) (■) and DSPC/Chol (pH_i 7.5) (s). Error bars represent the means and standard deviations from four mice.

exposed on the exterior surface of the liposome (i.e., pH_i 7.5) was not eliminated as rapidly as cationic liposomes prepared using diacyl cationic lipids [11]. This may be due, in part, to loss of the monoacyl SPH from the liposomes following administration; a lipid exchange *in vivo* that would not be observed for diacyl cationic lipids. The slower liposomal clearance observed in Fig. (**1B**), compared to that in Fig. (**3**), is due to the fact that the liposomes presented in Fig. (**1B**) were loaded with vincristine but those in Fig. (**3**) were empty. Slower clearance rates are commonly observed when the liposomes are loaded with cytotoxic agents, compared to empty liposomes, due to the ability of the encapsulated drug to "poison" mononuclear phagocytic system (MPS) cells involved in liposome clearance. This characteristic has been referred to in the literature as MPS blockade [29].

Anti-tumor Activity of Liposomal Vincristine

Previous studies have shown that increased vincristine retention within liposomes significantly improves anti-tumor activity [5-7]. To determine if this relationship existed in SPH-containing formulations, the anti-tumor activity of the vincristine formulation of DSPC/Chol/SPH pH_i 2.0 formulation was compared to that of DSPC/Chol pH_i 2.0 and DSPC/Chol pH_i 4.0. For these studies we used the murine P388 lymphocytic leukemia model that has been used frequently in the past to rapidly assess the activity of vincristine and to differentiate the therapeutic activity of different liposomal formulations of vincristine [5, 23, 30]. Results obtained in this model provide a foundation by which others can compare the results reported here to previously described formulations. As summarized in Table **1**, all liposomal formulations of vincristine were significantly more efficacious than free vincristine against the P388 tumor. This is shown (Table **1**) by increases in median survival times and % ILS and is best highlighted by the increases in the L/F ratio (median survival time for the liposomal vincristine group/median survival time for the equivalent dosage of free drug) in the liposomal groups.

The results presented in Table **1** also clearly show that the DSPC/Chol/SPH (pH_i 2.0) formulation had greater activity against the P388 tumor than either the DSPC/Chol (pH_i 2.0) or the DSPC/Chol (pH_i 4.0) formulation. Specifically, at the well-tolerated dose of 2 mg/kg vincristine, the median survival times were 27, 31 and 36 days for DSPC/Chol (pH_i 4.0), DSPC/Chol (pH_i 2.0) and DSPC/Chol/SPH (pH_i 2.0) liposomal vincristine formulations respectively. These median survival times were statistically different ($p < 0.01$) are represented corresponding %ILS values of 170, 210 and 260 (Table **1**). The DSPC/Chol/SPH (pH_i 2.0) liposomal vincristine given at 3 mg vincristine/kg produced long-term (60%) survivors and had a median survival time of > 60 days. In contrast, DSPC/Chol (pH_i 4.0) liposomal vincristine produced no long-term survivors at this dose and the DSPC/Chol (pH_i 2.0) formulation had only 40% long-term survivors and a shorter median survival time (36 days) (Table **1**). All free and liposomal vincristine formulations administered at 4 mg/kg resulted in mean body weight decreases in excess of 15% (data not shown). Therefore, these doses were higher than the maximum tolerated dose and efficacy comparisons between the formulations were not conducted. However, the data presented in Table **1** suggests that the effective retention of vincristine in DSPC/Chol/SPH (pH_i 2.0) liposomes reduced the systemic toxicity, as judged by drug-induced weight loss, compared to that of the DSPC/Chol (pH_i 4.0) and DSPC/Chol (pH_i 2.0) formulations with more rapid drug leakage.

Previous studies of liposomal anticancer agents [5, 9, 23, 30-33], including liposomal vincristine formulations [5, 7, 30, 32, 33], have shown that improved drug retention and drug concentrations in the plasma are associated with increased anti-tumor activity. This report also emphasizes the strong causal correlation between improved vincristine

Table 1. Activity of Free and Liposomal Vincristine Against the P388 Leukemia Tumor in BDF1 Mice

Sample	Dose (mg/kg)		Wt. change on Day 7 (%)	Survival		ILS[1] (%)	L/F[2]
	Vincristine	Lipid		At 60 Days	Median (days)		
Saline (control)	-	-	+ 14.4	0/15	10	-	-
Free vincristine	2.0	-	+6.0	0/10	14	40	-
Free vincristine	3.0	-	-3.6	0/10	12	20	-
DSPC/Chol + vincristine (pH_i 4.0)	2.0	20	-2.1	0/10	27	170	1.93
DSPC/Chol + vincristine (pH_i 4.0)	3.0	30	-12.0	0/10	31	210	2.58
DSPC/Chol + vincristine (pH_i 2.0)	2.0	20	0.0	2/5	31	210	2.21
DSPC/Chol + vincristine (pH_i 2.0)	3.0	30	-10.2	2/5	36	260	3.00
DSPC/Chol/sphingosine + vincristine (pH_i 2.0)	2.0	20	+1.8	1/5	36	260	2.57
DSPC/Chol/sphingosine + vincristine (pH_i 2.0)	3.0	30	-7.0	3/5	> 60	ND[3]	ND[3]

[1]Percentage of Increase of Life Span (%ILS) values were determined from median survival time comparing treated and saline control groups.

[2]L/F (Liposomal/Free) values were calculated by dividing the median survival time for the liposomal vincristine group by the median survival time for the equivalent dosage of free drug.

[3]N.D. = Not Determined. Median survival times and %ILS were not calculated if greater than 50% of the animals survived for greater than 70 days.

retention within liposomes in the DSPC/Chol/SPH (pH_i 2.0) formulation and decreased vincristine elimination from the circulation, with improved therapeutic activity against the P388 leukemia (Table **1**). However, it is also possible that some of the observed therapeutic activity (Table **1**) was due to combination therapy achieved by the co-administration of SPH and vincristine. It is well established that sphingolipids, including ceramides and sphingosine, have messenger functions that regulate the proliferation, survival, and death of cells [34]. While emphasis has been placed on the development of ceramides and their derivatives as anticancer agents [35, 36], apoptosis-promoting activity has also been observed in sphingosine and its derivatives [37]. Thus, we are exploring the potential of using therapeutically active lipids, in combination with liposome-encapsulated drugs, to define effective drug combinations for use in the treatment of cancer. We are now pursuing further studies with SA- and SPH-containing liposomes as agents that can enhance the activity of anti-cancer drugs such as vincristine through alterations in drug retention, facilitation of accumulation at tumor-associated vascular endothelium and which may have their own cytotoxic activity. Future studies will be conducted in solid human xenograft models (i.e. the HT-29 human colorectal model) to more accurately model tumor neovasculature relevant to the clinical setting.

Finally, we cannot discount the possibility that the cationic liposomes may foster interaction with tumor-associated vascular endothelial cells affected by P388 proliferation within the peritoneal cavity. It is known that hyper-permeability of blood vessels is associated with ascites tumors [38, 39] and that this may be a common consequence of secreted angiogenic factors such as VEGF [40, 41]. Hyper-permeability of tumor-associated blood vessels has been linked to increased exchange of macromolecules [38] and liposomes [42] between the blood and the peritoneal cavity in ascitic tumor-bearing mice. Since it has been established that cationic liposomes bind to angiogenic blood vessels [14-16, 43], it is possible that, over time after i.v. administration, the cationic lipids localized to the inner monolayer of the liposome redistribute to the outer leaflet. This redistribution may be sufficient to promote accumulation at affected blood vessels. We are now attempting to establish this more directly using immunohistochemical methods.

CONCLUDING REMARKS

In this study, significantly improved *in vivo* retention of the cationic drug, vincristine, was achieved using monoacyl cationic lipids SA and SPH that are capable of migrating to the inner monolayer of a liposomal membrane in response to a transmembrane pH gradient. Prolonged *in vivo* retention of vincristine in SA- or SPH-containing liposomes required both the cationic lipid and a low internal pH (pH_i 2.0). Enhanced retention of vincristine using SPH and a transmembrane pH gradient was associated with significant increases in anti-tumor activity against the murine P388 leukemia.

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