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Optimization of the retention properties of vincristine in liposomal systems

Nancy L. Boman [a,*], Lawrence D. Mayer [b] and Pieter R. Cullis [a]

[a] *The University of British Columbia, Biochemistry Department, 2146 Health Sciences Mall Vancouver, British Columbia V6T 1Z3 (Canada) and* [b] *British Columbia Cancer Agency, 600 West 10th Avenue, Vancouver, British Columbia V5Z 4E6 (Canada)*

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The influence of lipid composition, internal pH and internal buffering capacity on the retention properties of vincristine loaded into large unilamellar vesicle (LUV) systems in response to transmembrane pH gradients has been assessed. It is shown that increasing the (saturated) acyl chain length of the phosphatidylcholine molecule, increasing the internal buffering capacity, and decreasing the internal pH all result in increased drug retention. Further, a study of the pH dependence on the rates of accumulation indicate that uptake proceeds via the neutral form of the vincristine molecule. This uptake is associated with an activation energy of 37 kcal/mol for DSPC/Chol LUVs. It is shown that the major improvement in drug retention in vitro is achieved by employing low initial internal pH values, where 90% retention is obtained over 24 h for an initial internal pH of 2. Improved retention in vivo was also observed where a drug-to-lipid ratio approx. 4-fold greater at 24 h was maintained.

Introduction

Encapsulation of various antineoplastic agents within lipid vesicles has been shown to decrease toxic side effects while increasing or maintaining therapeutic activity [1–12]. This includes the vinca alkaloid vincristine [13]. Since the vinca alkaloids are cell-cycle-specific cytotoxic drugs, it may be conjectured that an ability to maintain high serum levels of drug for extended periods would be advantageous. A liposome system which allows extended payout in the circulation is therefore desireable. Previous work employing doxorubicin has shown that increased drug retention leads to an increase in drug circulation time [14] and increased antitumor activity [15]. However, currently available retention properties for vincristine are not optimal, as the best available formulation of liposomal vincristine releases drug in vivo with a half-life of less than 8 h, leading to more than 90% release by 24 h.

In this work we explore three parameters which may be expected to result in improved retention characteristics of liposomal formulations of vincristine and other lipophilic, amino-containing drugs. These include the use of phospholipids with increased acyl chain length, reduction of the interior pH and increased interior buffering capacity. The latter two parameters are important factors in the loading of lipophilic amines, such as vincristine, into vesicles exhibiting a transmembrane pH gradient (ΔpH; inside acidic) [13,16]. It is shown that the major factor resulting in improved retention is the interior pH, where initial interior pH values of 2 result in nearly 50% retention at 24 h in vivo.

Materials and Methods

Dimyristoyl PC (DMPC), dipalmitoyl PC (DPPC), distearoyl PC (DSPC), diarachidoyl PC (DAPC) and dibehenoyl PC (DBPC) were purchased from Avanti Polar Lipids, and were > 99% pure. Cholesterol and all salts were obtained from Sigma (St. Louis, MO, USA). Vincristine sulfate was purchased from the British Columbia Cancer Agency (Vancouver, British Columbia, Canada). [^{14}C]cholesteryl hexadecyl ether was purchased from New England Nuclear (Ontario, Canada) and was > 95% pure. It was chosen as a lipid marker due to its stability in vivo [17]. Tritiated vincristine was obtained from Amersham (Oakville, Ontario, Canada). Normal mouse serum was purchased from Cedar Lane Laboratories and female BDF1 mice (6–8 weeks old) were purchased from Charles River Laboratories.

PC/Chol (55:45 molar ratio) liposomes were prepared by first dissolving the lipid mixture in 95%

* Corresponding author. Fax: +1 (604) 822 4843.

ethanol at a lipid concentration of 100 mg/ml. Multilamellar vesicles (MLVs) were formed by adding citrate buffer to the ethanol solution to achieve a final concentration of 25 mg total lipid/ml. The samples were then maintained above the transition temperature for an additional 30 min to allow equilibration of buffer across the lipid bilayers (65°C for DMPC, DPPC and DSPC; 85°C for DAPC; and 100°C for DBPC). These MLVs were then extruded 10 times through an extrusion device obtained from Lipex Biomembranes (Vancouver, British Columbia, Canada) equilibrated at the same temperature [18]. The extruder was fitted with two Nucleopore polycarbonate filters with a pore size of 100 nm. Ethanol was then removed from the resulting LUVs by dialysis against two changes of 100 volumes of citric acid buffer over a 24-h period employing Spectra/Por 2 dialysis tubing (cutoff 12–14 kDa). This procedure has shown to remove > 99.9% of the ethanol from the liposomes (employing [^{14}C]EtOH).

For the vincristine uptake experiments, drug was added to the various liposome preparations to achieve a maximum drug-to-lipid ratio of 0.1 : 1. The exterior pH of the vesicles was then raised to pH 7.0–7.2 with 0.5 M Na_2HPO_4 and incubated at 37°C over a 4-h period. Aliquots were removed at various time points for determination of vincristine uptake. External untrapped vincristine was removed by running the samples over G-50 Sephadex columns prior to dual label scintillation counting of the liposomal fractions contained in the void volume.

Vincristine release experiments were performed as follows. Vincristine was entrapped in the liposomes using the same procedure as for the drug uptake experiments, except that the samples were immediately heated to their lipid transition temperatures for 10 min. This ensured > 95% trapping efficiencies at a drug-to-lipid ratio fo 0.1 : 1 for all lipid compositions studied [13].

The liposomally entrapped drug was then diluted in either HBS (pH 7.4) or normal mouse serum by a factor of 10. These samples were dialyzed using Spectra/Por 2 dialysis tubing against 200 vols. of HBS (pH 7.4) at 37°C. Aliquots were removed at various time points, run down G-50 Sephadex columns, and retained vincristine analyzed by dual label scintillation counting.

In vivo experiments were performed by injecting liposomal vincristine into BDF-1 mice via a lateral tail vein (2 mg/kg vincristine, 20 mg/kg lipid). At varying time points, mice were anaesthetized with i.p. ketamine (160 mg/kg) and xylazine (20 mg/kg). Blood was removed via cardiac puncture and placed into EDTA-coated microtainer tubes (Becton Dickenson). Samples were then centrifuged and plasma was analyzed for lipid and vincristine content by dual label liquid scintillation counting.

Kinetic analysis of vincristine uptake and release

An initial-rates treatment of the uptake of lipophilic amino containing drugs into vesicles with an acidic interior has been previously developed for doxorubicin [23]. We consider first weak bases with a single ionizable group with a dissociation constant (K_d) where $K_d \ll [H^+]_o$ where $[H^+]_o$ is the exterior proton concentration. Assuming that V_o, the aqueous volume, is much larger than V_m, where V_m is the volume of the membrane, it can be shown that

$$[D(t)]_i = [D(eq)]_i(1 - e^{-kt}) \quad (1)$$

where $[D(t)]_i$ is the interior concentration of drug at time t, $[D(eq)]_i$ is the interior drug concentration at equilibrium and k is the rate constant associated with uptake. The rate constant k can be written as [23]

$$k = \frac{PA_mK}{V_o[H^+]_o}K_d \quad (2)$$

where P is the membrane permeability coefficient for the neutral form of the weak base, A_m is the area of the membrane, and K is the lipid-water partition coefficient of the drug. It is straightforward to perform a similar analysis for a drug such as vincristine, which contains two basic functions. Under the assumption that $[H^+]_o \gg K_{d1}$, K_{d2} (where K_{d1}, K_{d2} are the dissociation constants associated with the two basic groups) it can be shown that

$$k = \frac{PA_mK}{V_o[H^+]_o^2}K_{d1}K_{d2} \quad (3)$$

As noted in Results, the condition $[H^+]_o \gg K_{d1}$, K_{d2} is difficult to observe for vincristine as the ionizable groups exhibit relatively low pK values of p$K_1 = 5.0$ and p$K_2 = 7.4$. Thus, at external pH values below 5 the rate constant associated with uptake would be expected to increase as the pH is raised in proportion to $[H^+]_o^{-2}$, whereas in the interval $5 < pH_o < 7.4$ it would be expected that $k \propto [H^+]_o^{-1}$.

Results

Influence of acyl chain length on vincristine retention

Previous studies on doxorubicin [19] and vincristine [13] have shown that drug retention after loading in response to a ΔpH (inside acidic) is enhanced in DSPC-cholesterol LUVs in comparison to EPC-cholesterol LUVs. It may be expected that the presence of longer chain saturated PCs will further improve the retention properties. The uptake and release properties of LUVs composed of diarachidoyl PC (DAPC) and dibehenoyl PC (DBPC), in combination with cholesterol, were therefore investigated.

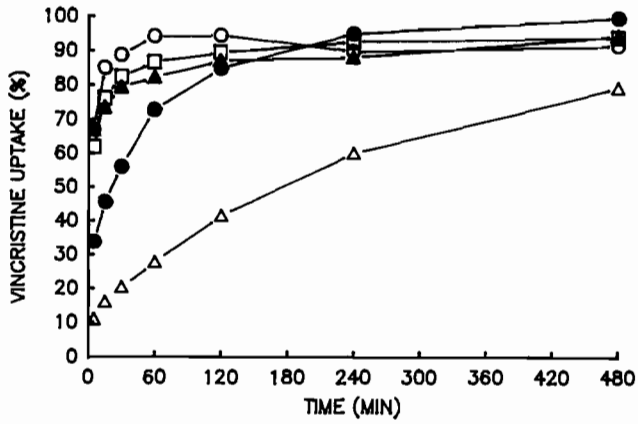

Fig. 1. Vincristine uptake across a pH gradient at 37°C for DMPC/Chol (○), DPPC/Chol (●), DSPC/Chol (△), DAPC/Chol (▲) and DBPC/Chol (□). Vincristine was added to vesicle preparations at a potential drug-to-lipid ratio of 0.1:1. Internal pH was 4.0 and external pH was 7.5. All vesicles were sized through 100-nm filters.

As a first step in this process, the production of LUVs containing the C_{20} compound DAPC and the C_{22} compound DBPC was required. Extrusion of DAPC dispersions, even in mixtures with cholesterol, is difficult at temperatures below the gel-liquid crystalline transition temperature of the DAPC (approx. 80°C). It was found that extrusion of DBPC/cholesterol (55:45) dispersions required an extrusion temperature in excess of 95°C, which proved experimentally inconvenient. The procedure detailed in Materials and Methods was therefore employed which involved extrusion in the presence of 25% ethanol, which markedly facilitated the ease of extrusion through 100 nm pore size. As indicated in Materials and Methods, the ethanol was subsequently removed by dialysis. For comparative purposes, LUVs for drug loading were prepared from mixtures of cholesterol with DMPC (C_{14}), DPPC (C_{16}), DSPC (C_{18}), DAPC (C_{20}) and DBPC (C_{22}). Standard procedures were employed, which involved hydration at pH 4.0 in the presence of 300 mM citrate, extrusion and ethanol removal as indicated in Materials and Methods and subsequently raising the exterior pH to 7.5 with 0.5 M Na_2HPO_4. The uptake of vincristine into these LUVs at 37°C is shown in Fig. 1. As expected, the rate of uptake was fastest for the DMPC-cholesterol system, and decreased progressively for DPPC-cholesterol and DSPC-cholesterol systems. Surprisingly, this progression was reversed for DAPC- and DBPC-cholesterol systems, which exhibited rates of vincristine uptake which increased as the acyl chain length increased.

The release characteristics of vincristine from the liposomes on prolonged incubation (24 h) at 37°C in the presence of buffer and serum were also investigated (results not shown). The DMPC-cholesterol system exhibits the most rapid leakage, whereas the DAPC-cholesterol and DBPC-cholesterol exhibit the best retention, with approx. 40% of the drug remaining at 24 h in the presence of mouse serum.

Influence of interior buffering capacity on vincristine retention

As has been well described elsewhere for doxorubicin [23], the ability to accumulate high levels of weak base drugs in response to ΔpH is a sensitive function of the interior buffering capacity. This is because the molecules permeate across the bilayer in the neutral form and are protonated on reaching the interior, thus consuming a proton and raising the interior pH. This will, in turn, limit the equilibrium uptake of drug. In the case of vincristine, for example, which contains two basic functions it is straightforward to show that, in the absence of membrane partitioning effects, and assuming that $[H^+]_o, [H^+]_i \gg K_{d1}, K_{d2}$ that

$$\frac{[\text{Drug}]_i}{[\text{Drug}]_o} \leq \frac{[H^+]_i^2}{[H^+]_o^2} \quad (4)$$

and thus the amount of drug entrapped will decrease as the square of the internal proton concentration as the internal pH rises.

The influence of interior buffering capacity on the retention properties of DBPC-cholesterol LUVs at 37°C was also investigated for both buffer and serum media (results not shown). As expected, higher initial internal citrate levels resulted in improved retention, however, these improvements were not significant for internal citrate concentrations in excess of 400 mM. At internal citrate concentrations of both 400 and 500 mM, >50% drug rentention was achieved at 24 h when incubated in mouse serum.

Influence of internal pH on drug retention

As indicated in Materials and Methods, a model based on the assumption that only the neutral form of the vincristine is membrane permeable predicts that the rate constant for transbilayer movement of vincristine should be proportional to the inverse square of the proton concentration. Thus, if the interior pH is lowered, the efflux of entrapped vincristine should be significantly slower. The first set of experiments was designed to examine the dependence of the rate constant k on the pH. As indicated in Materials and Methods, when $[H^+]_o \gg K_{d1}, K_{d2}$ it is expected that $k \propto [H^+]_o^{-2}$ and, thus, $\log k \propto 2\text{pH}_o$. Thus, a plot of $\log k$ vs. pH_o should result in a straight line with a slope of 2. Uptake behaviour was therefore monitored over the external pH range 4–5 for DSPC-cholesterol LUVs. As shown in Fig. 2A, the uptake rates vary dramatically over this range. The rate constants k can be calculated from the slopes of the semilogarithmic plots shown in Fig. 2B, leading to a plot of $\log k$ vs. pH_o (Fig. 2C) which exhibits a slope of 1.6. The devia-

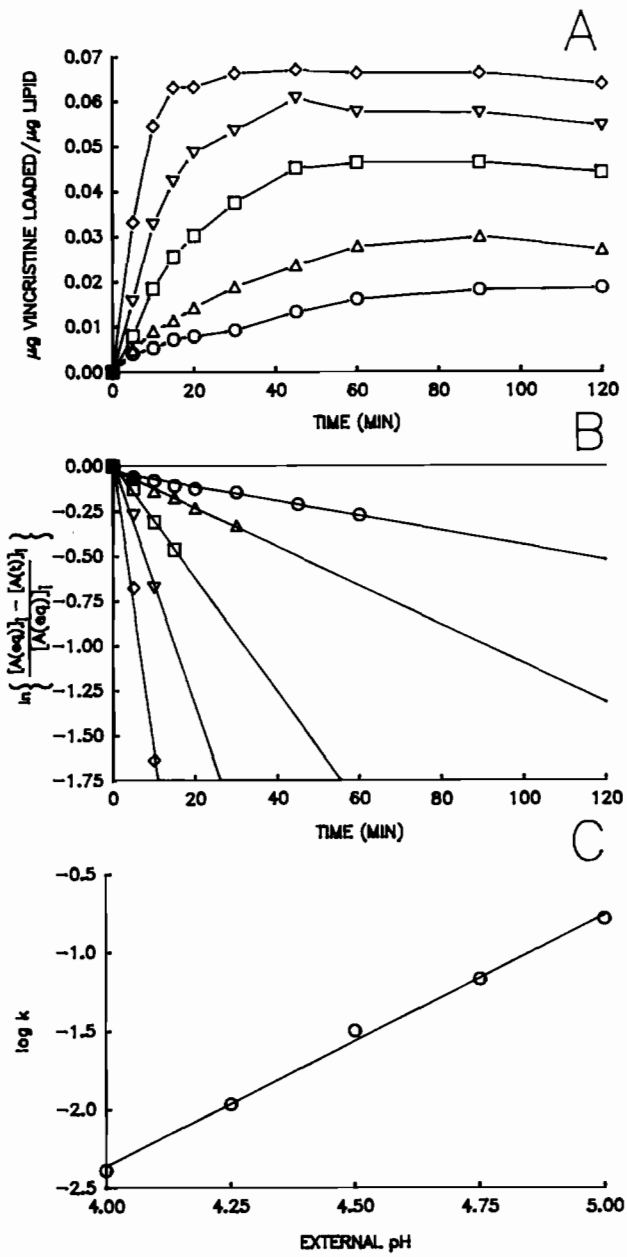

Fig. 2. (A) Time-course for vincristine uptake into 100-nm DSPC/Chol vesicles for different external pH values. The internal pH for all systems was 3.0. The external pH values were 4.00 (○), 4.25 (△), 4.50 (□), 4.75 (▽), and 5.00 (◇). All samples were loaded at 60°C with a potential drug-to-lipid ratio of 0.1:1. (B) Plot of $\ln\{([A(\mathrm{eq})]_i - [A(t)]_i)/[A(\mathrm{eq})]_i\}$ vs. time, where $[A(t)]_i$ is the internal concentration of the accumulated drug at time t and $[A(\mathrm{eq})]_i$ is the internal concentration at equilibrium. The slopes of these lines give the rate constant (k) for the transport of vincristine across the liposome membrane. (C) Plot of log k vs. external pH. The slope of this line is 1.60.

tion from the slope of 2 expected is likely due to the low value of the first pK of vincristine (pK_1 = 5.0), and thus the condition $[H^+]_o \gg K_1$ is not well observed.

The results of Fig. 2 indicate that efflux rates will be extremely sensitive to the interior pH after loading vincristine, and that lower internal pH values should markedly increase retention. DSPC/cholesterol (55:45) LUVs were therefore prepared with initial interior pH values of 2.0, 3.0, 4.0 and 5.0, employing the 300 mM citrate buffer. As shown in Fig. 3, these variations had a profound effect on vincristine retention, with (initial) pH_i values of 3 or less, giving rise to essentially complete retention of contents for 24-h incubations in the presence of both buffer and serum. Lowering the internal pH appears to exhibit its greatest effect on improving drug retention initially. The slopes of the curves in Fig. 3 are not as markedly different after the 1 h time point.

Influence of temperature on vincristine uptake

A final variable which would be expected to influence vincristine uptake (and, by extension, release) is temperature. It has been shown elsewhere that weak bases such as doxorubicin (unpublished data), as well as amino acid and peptide derivatives [20] can exhibit high activation energies for uptake rates in the range of 30 kcal/mol. An activation energy of 30 kcal/mol corresponds to an uptake rate which increases by ap-

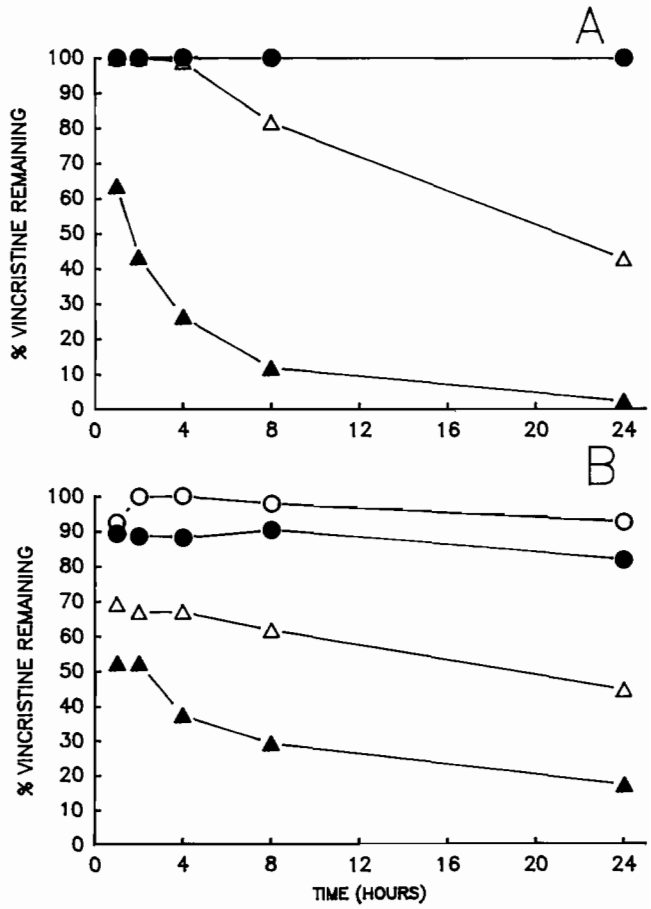

Fig. 3. Vincristine release from 100-nm DSPC/Chol vesicles incubated in buffer (A) and mouse serum (B) at 37°C for internal pH of 2.0 (○), 3.0 (●), 4.0 (△) and 5.0 (▲). Internal buffering capacity was 300 mM citrate for all systems. Initial drug-to-lipid ratios were 0.1:1.

prox. a factor of 5 for every 10°C increase in temperature. Vincristine uptake into DSPC-cholesterol LUVs was therefore monitored over the temperature range 30–60°C which resulted in remarkable differences in uptake rates as shown in Fig. 4A. An Arrhenius plot (Fig. 4C) of the rate constants derived from these data resulted in an activation energy of 37 kcal/mol.

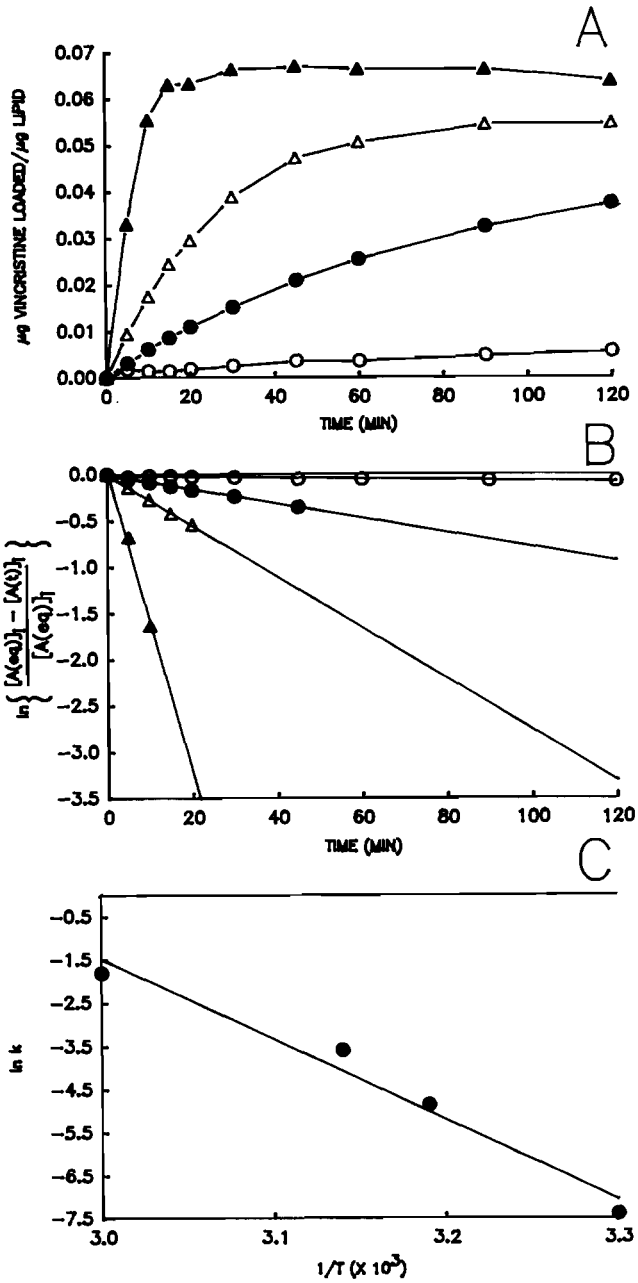

Fig. 4. (A) Time-course of vincristine uptake into 100-nm DSPC/Chol vesicles exhibiting a ΔpH ($pH_i = 3.0$; $pH_o = 5.0$). Uptake was conducted at 30 (○), 40 (●), 45 (△), and 60°C (▲). (B) Plot of $\ln\{([A(eq)]_i - [A(t)]_i)/[A(eq)]_i\}$ vs. t, where $[A(t)]_i$ and $[A(eq)]_i$ are the same as for Fig. 2. (C) Arrhenius plot of the rate constants (k) for vincristine uptake. The activation energy calculated from the slope of this plot is 37 kcal/mol.

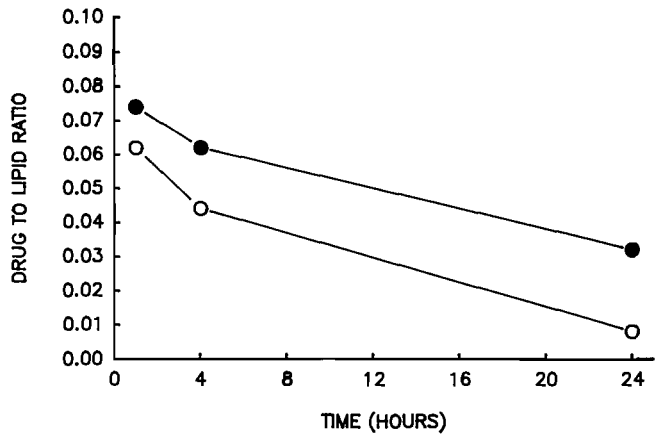

Fig. 5. Drug-to-lipid ratios for DSPC/Chol vesicles in vivo with internal pH of 2.0 (●) and 4.0 (○). Both systems were loaded with an initial drug-to-lipid ratio of 0.1:1. Each point represents the average value obtained from four BDF1 mice.

Vincristine retention in DSPC/cholesterol LUVs in vivo

A basic aim of these studies was to identify parameters which would lead to a formulation of liposomal vincristine which is able to better retain the drug in vivo to allow for extended circulation lifetime and payout characteristics. It is clear from the studies presented above that the internal pH is the most important variable for retention, and that DSPC/cholesterol LUVs prepared with an (initial) pH_i of 3 or less exhibit retention of 90% or more over 24 h in the presence of buffer or serum. However, it is also known that liposome leakage in vivo is usually more extensive than in vitro [21]. The release properties in vivo, of DSPC-cholesterol LUVs with (initial) interior pH values of 2 and 4 and loaded with vincristine were assessed by monitoring the drug-to-lipid ratio in plasma (Fig. 5). It may be observed that whereas the 90% retention over 24 h obtained in vitro was not achieved for the $pH_i = 2$ formulation, a value of 40% was achievable. This is approx. a factor of 5 higher than obtained with the $pH_i = 4.0$ formulation. It is once again apparent, as in vitro, that the most marked difference in vincristine release rates is seen before 1 h post-injection. It is of interest that DBPC/cholesterol (55:45) preparations with 0.5 M internal citrate at $pH_i = 2.0$ did not result in improved retention in vivo over the DSPC-cholesterol systems (results not shown). It should be noted that > 99% of injected free vincristine (no liposomal carrier) is cleared from the circulation within 5 min post injection (Mayer et al., unpublished data).

Discussion

This report presents a detailed study of factors leading to improved retention of vincristine in liposomal systems. These results may also be expected to extend to other members of the large class of drugs

which are lipophilic weak bases. Here we discuss the influence of the experimental parameters investigated on vincristine uptake and release and the implications for design of liposomal formulations of lipophilic amino containing drugs.

Increases in acyl chain length are shown to exhibit the type of retention improvements expected. Thus, the half-times for vincristine release at 37°C in buffer increase from approx. 1 h for DMPC/Chol LUVs to approx. 12 h for DAPC- and DBPC-containing systems. The uptake of vincristine into these systems exhibits anomalous behavior in that the rates of uptake first decrease, as expected, as acyl chain length is increased to 18 carbons (DSPC), and then increase markedly for the DAPC and DBPC systems. It is possible that this reflects an increased lipid-water partition coefficient for vincristine for the outer monolayer of the DAPC and DBPC systems. An increased hydrophobicity of this interface would be consistent with packing effects expected for longer chain lipids in small vesicular systems, and this is reflected by the increased tendency of the longer chain DAPC and DBPC LUV systems to aggregate after extrusion. Conversely, for the inner monolayer, it would be expected that these effects would result in tighter packing in the headgroup region, and correspondingly reduced leakage, as shown experimentally.

With regard to interior buffer capacity, the results presented here show that increasing the interior citrate concentration above 400 mM does not result in significant improvements in vincristine retention. This is consistent with the osmotic properties of extruded LUV systems. As shown for 100 nm egg PC/cholesterol systems [23], extruded LUVs exhibit tubular 'sausage' shapes and respond to osmotic gradients (high interior osmolarity) first by 'rounding up' to increase the interior volume and subsequently undergo osmotically induced lysis. For EPC/Chol LUVs the 'effective' osmotic difference that can be sustained is approx. 650 mosmol/kg. The osmolarity of 400 mM citrate, pH 4.0, is 700 mosmol/kg, resulting in an effective osmotic imbalance of 400 mosmol/kg when the liposomes are in normal saline solution (osmolarity 300 mosmol/kg). This initial effective osmotic imbalance will increase on drug loading. Vincristine, after crossing the liposomal bilayer in its neutral form, becomes protonated in the vesicle interior due to the low internal pH, consequently raising the internal osmolarity.

Lower internal pH values are clearly critical for improving vincristine retention in liposomal systems. There are two factors which lead to this effect. The first involves the improved internal buffering capacity resulting from initial interior pH values which are below the lowest pK ($pK_1 = 3.13$) of the citrate carboxyl functions. However, the dominant effect is likely due to the dependence of the rate constant for vincristine movement on the inverse square of the proton concentration (Eqn. 3). This predicts a 100-fold reduction in leakage rates for every unit the interior pH is lowered.

In conclusion, the internal pH is the most important parameter in enhancing liposomal vincristine retention both in vitro and in vivo. These effects can likely be extended to enhance retention of other basic drugs in liposomes in vitro and in vivo.

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