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Therapeutically optimized rates of drug release can be achieved by varying the drug-to-lipid ratio in liposomal vincristine formulations

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Abstract

The anti-tumor efficacy of liposomal formulations of cell cycle dependent anticancer drugs is critically dependent on the rates at which the drugs are released from the liposomes. Previous work on liposomal formulations of vincristine have shown increasing efficacy for formulations with progressively slower release rates. Recent work has also shown that liposomal formulations of vincristine with higher drug-to-lipid (*D/L*) ratios exhibit reduced release rates. In this work, the effects of very high *D/L* ratios on vincristine release rates are investigated, and the antitumor efficacy of these formulations characterized in human xenograft tumor models. It is shown that the half-times ($T_{1/2}$) for vincristine release from egg sphingomyelin/cholesterol liposomes *in vivo* can be adjusted from $T_{1/2} = 6.1$ h for a formulation with a *D/L* of 0.025 (wt/wt) to $T_{1/2} = 117$ h (extrapolated) for a formulation with a *D/L* ratio of 0.6 (wt/wt). The increase in drug retention at the higher *D/L* ratios appears to be related to the presence of drug precipitates in the liposomes. Variations in the *D/L* ratio did not affect the circulation lifetimes of the liposomal vincristine formulations. The relationship between drug release rates and anti-tumor efficacy was evaluated using a MX-1 human mammary tumor model. It was found that the antitumor activity of the liposomal vincristine formulations increased as *D/L* ratio increased from 0.025 to 0.1 (wt/wt) ($T_{1/2} = 6.1$ –15.6 h respectively) but decreased at higher *D/L* ratios (*D/L* = 0.6, wt/wt) ($T_{1/2} = 117$ h). Free vincristine exhibited the lowest activity of all formulations examined. These results demonstrate that varying the *D/L* ratio provides a powerful method for regulating drug release and allows the generation of liposomal formulations of vincristine with therapeutically optimized drug release rates.

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Keywords: Liposome; Vincristine; Regulated drug release; Efficacy; Drug precipitation

1. Introduction

Liposomal formulations of anticancer drugs can result in increased activity and reduced toxicity compared to the free drug [1–5]. The benefits of liposomal encapsulation are, however, dependent on the rates of drug release from the liposomes, leading to the need to develop formulations that exhibit thera-

apeutically optimized drug release rates. Such therapeutically optimized release rates have not been developed for any liposomal formulation of any drug to date. The major focus of research efforts has been on developing liposomal formulations that are small (diameter ~100 nm) and long circulating in order to take advantage of the “enhanced penetration and retention” (EPR) effect [6] leading to preferential accumulation of the liposomes at tumor sites and infection to sites of infection and inflammation due to the hyperpermeable nature of the vasculature in these regions [7]. Within the context of these formulations, there is a clear need to keep the drug encapsulated during the time it takes to achieve appreciable tumor accumulation of the carrier, which implies a half-time for drug

Abbreviations: SM, Egg sphingomyelin; [³H]-CHE, [³H]-cholesterylhexadecyl ether; MLV, multilamellar vesicles; LUV, large unilamellar vesicles; FBS, fetal bovine serum; *D/L* ratio, drug-to-lipid ratio

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release ($T_{1/2}$) of hours or longer. However, there is an equally clear need for the drug to be released on arrival at the tumor site at rates sufficient to provide enough bio-available drug to inhibit tumor growth.

Previous work has shown that the efficacy of liposomal formulations of cell cycle-specific drugs such as vincristine is particularly sensitive to drug release rates. For example, in studies employing the L1210 and P388 murine leukemia models, or the A431 human squamous cell carcinoma model, formulations of vincristine with progressively slower release rates converted the drug from being essentially inactive to one that produced 100% cures [8,9]. This dependence on release rates is consistent with the fact that prolonged exposure of cells to cell cycle-specific agents results in greater cell killing in vitro and enhanced antitumor activity in vivo [10–15]. Thus, prolonged vincristine release from liposomes accumulated at the tumor site and the resulting extended tumor cell exposure would be expected to result in enhanced antitumor activity. However, formulations with release rates so slow that efficacy is compromised have not yet been achieved, preventing identification of truly optimized release rates.

Considerable efforts have been made to develop liposomal vincristine formulations with improved retention and efficacy properties. These include increasing acyl chain length and saturation [16], using sphingomyelin (SM) instead of phosphatidylcholine [17], and using lower internal pH environments in the liposome [18–20]. An SM/Chol liposome formulation [21] was identified as having the best retention properties achievable ($T_{1/2}$ in vivo of ~24 h) using this technology, and this formulation has shown enhanced efficacy profiles both in animal models and in humans [22–26].

In recent work, we have shown that the release rates of vincristine and other drugs from liposomes are highly sensitive to the drug-to-lipid (D/L) ratio employed in the formulation [27]. In the case of vincristine an increase in the D/L ratio from 0.1 to 0.3 (wt/wt) results in an increase in the half-time for drug release of more than a factor of two. Here, we explore the possibility that, at higher D/L values achievable through the use of an ionophore-based drug accumulation technique [28], rates of drug release can be so slow that a therapeutically optimized release rate can be identified. In addition, studies to determine the mechanism whereby increased D/L values lead to improved retention have also been performed; with particular attention paid to the possibility that high D/L values lead to precipitation of the drug in the liposome interior.

2. Materials and methods

2.1. Materials

Egg sphingomyelin (SM) was purchased from Northern Lipids (Vancouver, BC, Canada) and was 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. [^3H]-Cholesterylhexadecyl ether (CHE) was obtained from Perkin Elmer Life Sciences (Boston, MA, USA). [^{14}C]-vincristine sulfate was obtained from Chemsyn Laboratories (Lenexa, KS, USA). All other chemicals were obtained from Sigma (St. Louis, MO, USA).

2.2. Liposome preparation

Lipids (SM or Chol) and trace amounts of [^3H]-CHE were co-dissolved at appropriate molar ratios in ethanol. Multilamellar vesicle (MLV) suspensions were generated after the addition of a 300-mM aqueous solution of magnesium sulfate, yielding a final ethanol concentration of 10% (v/v). Large unilamellar vesicles (LUVs) 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) [29,30]. 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). The mean diameter of LUVs was determined by dynamic light scattering using a NICOMP 370 particle sizer (Nicomp Particle Sizing Inc., Santa Barbara, CA) and found to be 110 ± 25 nm. Phospholipid concentrations were determined using established techniques [31] and the specific activity of the liposomes was determined using a Beckman LS3801 scintillation counter (Fullerton, CA, USA).

2.3. Vincristine encapsulation in liposomes

Vincristine was encapsulated into LUVs using an ionophore-mediated drug loading procedure, as described previously [28]. Briefly, vincristine sulfate and trace amounts of [^{14}C]-vincristine sulfate were added to LUVs (5 mm final lipid concentration) at appropriate drug-to-lipid ratios (wt/wt), and subsequently pre-incubated at 65 °C prior to the addition of the calcium ionophore A23187. The LUV/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 [28] prior to quantification of liposome entrapped drug using dual channel counting on a Beckman LS 3801 scintillation counter. Formulations used for in vivo studies received additional dialysis into 300 mM sucrose prior to quantification of liposome-entrapped drug.

2.4. In vitro release of vincristine

In vitro drug release assays were performed to make quantitative comparisons of drug leakage between formulations of varying drug-to-lipid ratios. Initially, in vitro release assays for liposomal vincristine were conducted using ammonium chloride to degrade the pH gradient [32]. 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 20–30% after 60 min for a drug-to-lipid ratio of 0.05 (wt/wt). Leakage of vincristine from the loaded LUVs was assayed by the removal of aliquots for spin column analysis and quantification using dual label liquid scintillation counting [28,33]. Vincristine release from liposomes was also assessed using fetal bovine serum. Liposomal drug in SEH loading buffer was added to fetal bovine serum (FBS; Invitrogen, San Diego, CA) to give a final total lipid concentration of 1.25 mM and a final FBS concentration of 50% (v/v). Samples were incubated at 50 °C. Vincristine release was quantified over time through the removal of aliquots for spin column analysis and dual label liquid scintillation counting. 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. Error bars were calculated from the 95% confidence intervals associated with the exponential best fits.

2.5. Mice

Female, 6- to 8-week-old outbred ICR mice were obtained from Harlan (Indianapolis, IN) and were used for pharmacokinetic and drug leakage studies. Female, 6–8 week old, athymic CrI:CD-1[®]-nuBR mice were obtained from Charles River Laboratories (Quebec, Canada) and used for the antitumor efficacy studies. All mice were quarantined for at least 2 weeks prior to use. Immunocompromised animals were maintained under sterile conditions, with a controlled temperature (22 ± 1 °C) and humidity ($60 \pm 10\%$) 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).

2.6. *In vivo* pharmacokinetics

Vincristine loaded LUVs 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 drug and lipid doses of 2 and 40 mg/kg, respectively, in mice. 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. In some instances, formulations with different *D/L* ratios were injected into mice at equivalent lipid doses (no empty liposomes) to determine whether formulations with high drug-to-lipid ratios exhibited altered pharmacokinetics. 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 $400 \times g$ for 15 min and plasma was collected and decolorized for lipid and drug determination by dual label liquid scintillation counting as described previously [28].

2.7. *In vivo* efficacy studies

MX-1 human mammary adenocarcinoma was obtained from the Division of Cancer Treatment and Diagnosis (DCTD) Tumor Repository (Frederick, MD), maintained by serial passage *in vivo* and implanted by trocar into the dorsal flank of nude mice. Experiments were performed between the fourth and tenth serial passage in nude mice. Treatments were initiated in all models when tumor volumes were 200–300 mm³ (14 days post tumor implantation). Animals were dosed according to body weight (10 ml/kg body weight) and received a single intravenous (*i.v.*) injection of one of several formulations of liposomal vincristine. Liposomal vincristine formulations were diluted to achieve a total lipid dose of 66 mg/kg and a drug dose of 1.5 mg/kg body weight. As with the pharmacokinetic studies, empty liposomes were included, as appropriate to ensure mice received equivalent total lipid doses for all drug-to-lipid ratios. Tumors were measured at least three times per week with calipers and tumor volume (mm³) was calculated using the formula: $(\text{length} \times \text{width}^2)/2$, where width was the smaller of the two perpendicular measurements [34]. Tumor growth delay ($T - C$); the median difference in time (days) for treated and control tumors to reach 1000 mm³, was evaluated for each treatment group.

2.8. Cryo-transmission electron microscopy

Cryogenic transmission electron microscopy (cryo-TEM) was performed on empty and drug-loaded SM/Chol liposomes using a Zeiss EM 902A Transmission Electron Microscope (LEO Electron Microscopy, Oberkochen, Germany) operated at 80 kV in the zero loss bright-field mode. Digital images were recorded under low dose conditions with a BioVision Pro-SM Slow Scan CCD camera (Proscan GmbH, Scheuring, Germany) and analySIS software (Soft Imaging System, GmbH, Münster, Germany). In order to visualize maximum detail, an underfocus of 1–2 μm was used to enhance the image contrast. Sample preparation was performed at 25 °C and approximately 99% relative humidity within a climate chamber. A small drop (ca 2 μl) of sample was deposited on a copper grid covered with a perforated polymer film coated with carbon on both sides. Excess liquid was removed by blotting with filter paper, leaving a thin film of the solution on the grid. Immediately after blotting, the sample was vitrified by plunging the grid into liquid ethane held at -182 °C. Samples were maintained below -165 °C and protected against atmospheric conditions during both transfer to the TEM and examination. Images at 100,000 \times total magnification were captured for each sample.

3. Results

3.1. Vincristine can be loaded into liposomes to high drug-to-lipid ratios employing the ionophore loading technique

Previous work [27] has shown that vincristine loaded into LUVs using an ionophore loading technique exhibits improved retention at higher *D/L* values. For example, increasing the *D/L*

value from 0.1 to 0.3 (wt/wt) resulted in an increase in the half-time for retention by more than a factor of two. Initial work was therefore devoted to characterizing this effect further and, in particular, extending the range of *D/L* ratios that could be achieved. SM/Chol (55/45 mol) LUVs were prepared containing 300 mM MgSO₄ as described in Materials and methods and incubated in the presence of increasing amounts of vincristine corresponding to *D/L* ratios, if all the drug was accumulated, of up to 1.5. The LUVs were then dialyzed at room temperature for 2 h to remove untrapped drug and the *D/L* ratio measured as described in Materials and methods. As shown in Fig. 1, the efficiency of the accumulation process in the presence of the ionophore A23817 decreased at the higher vincristine concentrations, however *D/L* ratios as high as one (wt/wt) were achieved with approximately 50% trapping efficiency at the highest external vincristine concentrations employed.

3.2. The half-time of vincristine release from LUVs is linearly dependent on the drug-to-lipid ratio *in vitro* and *in vivo*

The next set of experiments were aimed at characterizing the release characteristics of SM/Chol LUVs loaded to achieve *D/L* ratios of up to 0.6 (wt/wt). Two *in vitro* assays were employed that resulted in release rates occurring on experimentally convenient timescales. The first involved incubation in the presence of 2 mM NH₄Cl at 50 °C, where the presence of the NH₄Cl raises the interior pH of the LUVs, converting more of the encapsulated drug to the neutral, membrane permeable form. The second assay involved incubation in the presence of 50% fetal bovine serum at 50 °C. Increased leakage in this case arises from adsorption of serum proteins to the LUV, thus increasing the permeability of the lipid bilayer. The high temperature was employed because incubation at 37 °C did not result in sufficiently rapid release characteristics. As shown in Fig. 2, both assays showed dramatic reductions in release rates as the *D/L* ratio is increased, corresponding to increases in the

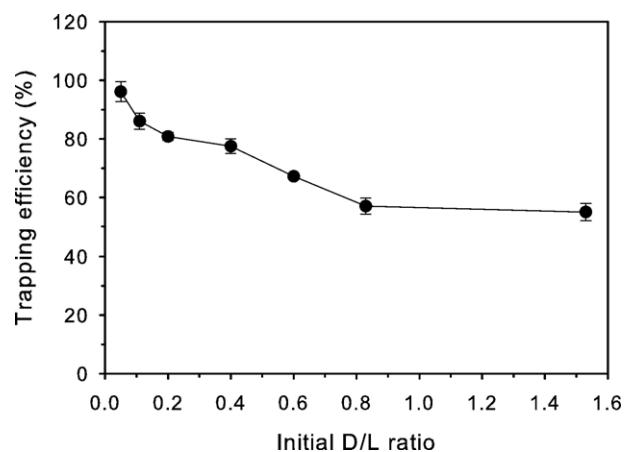


Fig. 1. Trapping efficiencies for formulations of liposomal vincristine at increasing initial drug-to-lipid ratios. Vincristine was loaded using the ionophore method, described in Materials and methods, into SM/Chol liposomes (55/45 mol%). Data points represent mean loading efficiencies (\pm standard deviations) calculated from 3 samples.

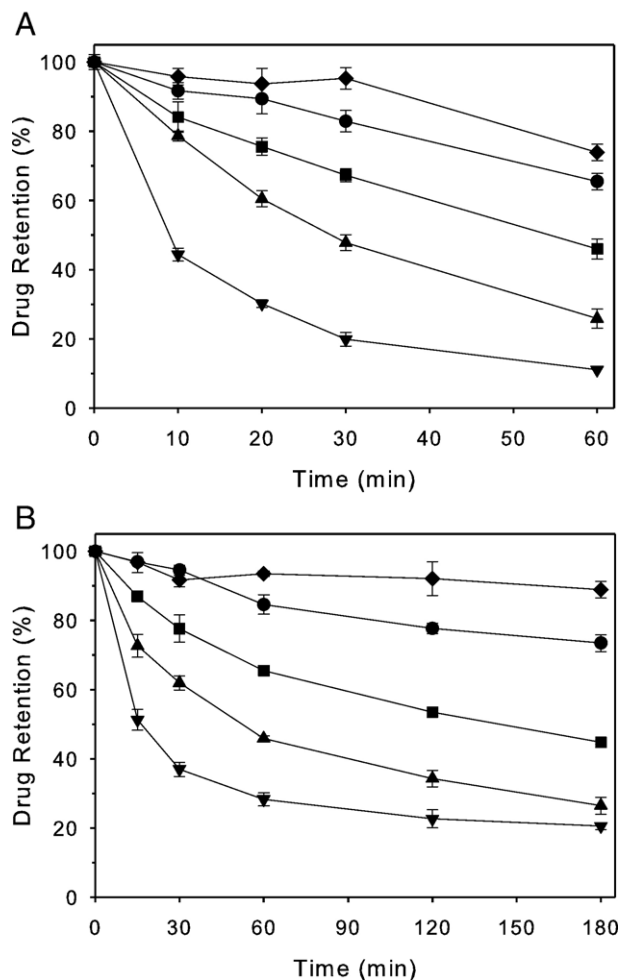


Fig. 2. Vincristine retention in SM/Chol (55/45, mol) liposomes in vitro. Panel A: retention in 2 mM ammonium chloride at 50 °C; formulations had drug-to-lipid ratios (wt/wt) of 0.05 (▼), 0.1 (▲), 0.2 (■), 0.35 (●) and 0.64 (◆). Panel B: retention in 50% FBS at 50 °C; formulations had drug-to-lipid ratios (wt/wt) of 0.05 (▼), 0.1 (▲), 0.2 (■), 0.4 (●) and 0.7 (◆). The lipid concentration in the release assays was 1.25 mM total lipid. Data points represent mean drug retention (\pm standard deviations) calculated from 3 samples and are representative of at least 3 separate experiments.

half-time of release of more than 10-fold as the D/L ratio is increased from 0.05 to 0.6 (wt/wt).

The drug retention properties of liposomal vincristine formulations with different D/L ratios were also evaluated in vivo. Outbred ICR mice were injected intravenously with liposomal vincristine formulations with D/L ratios of up to 0.33 at a dose corresponding to 2 mg/kg vincristine, which is the maximum tolerated dose of the free drug [8]. Since the total lipid dose can strongly affect the pharmacokinetic properties of liposomes [35], empty SM/Chol liposomes were included with drug-loaded formulations having higher D/L ratios to ensure that mice received an equivalent lipid dose of 40 mg/kg in all situations. Blood was collected at appropriate time points and assayed for total vincristine and total lipid. Note that free vincristine is rapidly removed from the circulation with the half-time for clearance of free vincristine less than 5 min [9] and thus the total amount of vincristine in the blood overwhelmingly reflects liposome-encapsulated drug [36]. As shown in Fig. 3, a remark-

able increase in the retention properties of the LUVs is observed as the D/L ratio is increased, corresponding to an increase in $T_{1/2}$ from ~ 6 h for a D/L ratio of 0.025 to more than 60 h for a D/L ratio of 0.33.

If it is assumed that the efflux of vincristine from LUVs obeys Fick's law, which states that the efflux rate is proportional to the area of the LUV membrane and the concentration gradient of vincristine across the membrane, then it is straightforward to show that $[D_i(t)] = [D_i(0)]\exp(-kt)$, where $[D_i(t)]$ is the concentration of drug inside the LUV at time t , $[D_i(0)]$ is the interior concentration of drug at time 0 and k is the rate constant associated with the release process. It follows that $[D_i(t)] / [D_i(0)] = \exp(-kt)$ and thus that the % released over time should be independent of the initial interior drug concentration, which is in direct contradiction to the results shown in Figs. 2 and 3. In an effort to understand the mechanism involved, the $T_{1/2}$ values calculated from the release data of Fig. 2 (incubation in the presence of ammonium chloride) and 3 were plotted as a function of initial D/L ratio. As shown in Fig. 4, a linear relationship is observed, which is consistent with a large proportion of the encapsulated drug being in a precipitated (non-soluble) form (see Discussion). A linear correlation between D/L ratio and the half-time of drug retention was also observed when drug leakage was induced by addition of fetal bovine serum.

3.3. Addition of "empty" liposomes allows equivalent clearance rates to be achieved for LUVs with different D/L ratios

At low lipid doses, LUVs are increasingly rapidly cleared from the circulation [35]. Thus, in order for LUV loaded with drug at very high D/L ratios to exhibit long circulation lifetimes,

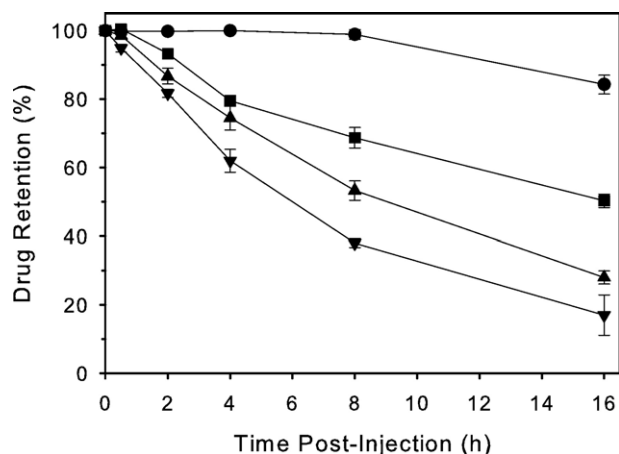


Fig. 3. Vincristine retention of SM/Chol (55/45, mol) liposomes in vivo. ICR mice were injected intravenously with SM/Chol formulations of vincristine having drug-to-lipid ratios (wt/wt) 0.025 (▼), 0.05 (▲), 0.1 (■) and 0.33 (●). All mice received lipid doses at 40 mg/kg total lipid and vincristine at 2 mg/kg drug, with the exception of the 0.025 D/L ratio sample which was dosed at 1 mg/kg vincristine. Rate constants for release were calculated to be 0.1128 ($T_{1/2}$ =6.1 h), 0.079 ($T_{1/2}$ =8.7 h), 0.0443 ($T_{1/2}$ =15.6 h) and 0.0106 h^{-1} ($T_{1/2}$ =65 h) for D/L ratios of 0.025, 0.05, 0.1 and 0.33, respectively. Data points represent mean values (\pm standard deviations) calculated from 3 mice.

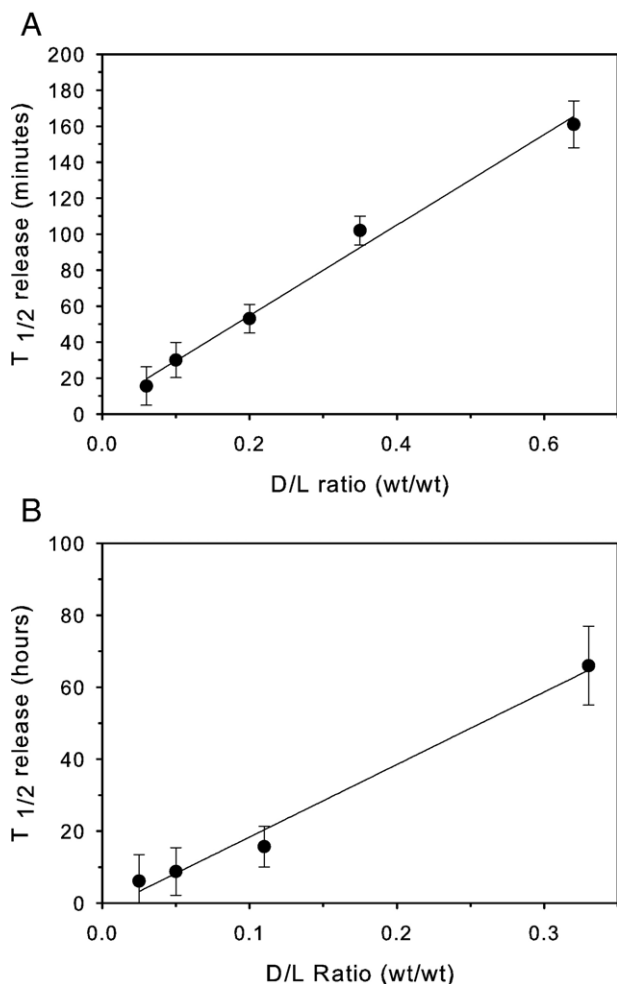


Fig. 4. Correlation of drug-to-lipid ratio and vincristine release half-life in ammonium chloride (Panel A) ($R^2=0.991$) and in female ICR mice (Panel B) ($R^2=0.994$). Half-lives were calculated as described in Materials and methods, and were 15, 30, 53, 102 and 161 min for D/L ratios of 0.06, 0.1, 0.2, 0.35 and 0.64 in vitro and 6.1, 8.7, 15.6 and 65 h for D/L ratios of 0.025, 0.05, 0.1 and 0.33 in vivo. Error bars were calculated from the 95% confidence interval associated with the best fit analysis.

it may be necessary to add “empty” liposomes to achieve the lipid dose required for long circulation lifetimes in combination with reasonable drug dose levels. For example, the maximum tolerated dose of vincristine is approximately 2 mg/kg in mice. If this dose was given in LUVs containing vincristine at a D/L ratio of one (wt/wt), this would correspond to a lipid dose of also 2 mg/kg, which is well below the lipid dose level at which dose-independent pharmacokinetics are observed for most LUV compositions [35]. However, as shown in Fig. 5, by adding empty liposomes to liposomal vincristine formulations with increasing D/L ratios to achieve constant lipid doses of 40 mg/kg, the circulation lifetimes following intravenous administration in vivo achieved are constant, with a half-time for clearance of approximately 8 h. Further, in order to show directly that high drug contents do not affect clearance behavior, the clearance behavior of LUVs with a D/L ratio of 0.4 (wt/wt) with no empty liposomes, administered at a vincristine dose of 20 mg/kg (corresponding to a lipid dose of 50 mg/kg), was also examined.

The clearance behavior of these systems was no different than other formulations (Fig. 5).

3.4. Liposomes containing high levels of vincristine show internal morphology consistent with drug precipitation

Structural and morphological changes in liposomes, attributed to precipitation of encapsulated drug, have been observed employing cryo-transmission electron microscopy (cryo-TEM) when certain drugs, such as doxorubicin, are loaded into liposomes [37]. In addition, previous studies have indicated that precipitation of encapsulated drug may enhance in vitro and in vivo drug retention [38]. Our previous cryo-EM studies on liposomal vincristine formulations [27] have shown that increasing internal electron densities are observed as the D/L ratio is increased to 0.3, however, no unambiguous evidence for drug precipitates was observed. Efforts were therefore made to produce liposomal vincristine formulations with the highest D/L ratios achievable in order to visualize potential internal structures. As shown above (Fig. 1), D/L ratios as high as 1 (wt/wt) can be achieved using the ionophore technique. Cryo-TEM studies were therefore conducted to include LUVs with these very high vincristine contents. As shown in Fig. 6, a progressive increase in intravesicular electron density as the D/L (wt/wt) ratio is increased from 0 to 1.0 is observed (Fig. 6, panels A–E). This increase in electron density could represent an amorphous or gel-like precipitate at the lower vincristine contents. However, at the D/L ratio of 1.03 (wt/wt), a large proportion of the liposomes contains electron dense granular structures within the vesicles (Fig. 4, panel F). These structures, which are unlike the more linear precipitates observed for drugs such as doxorubicin and topotecan [38,39], represent the first direct

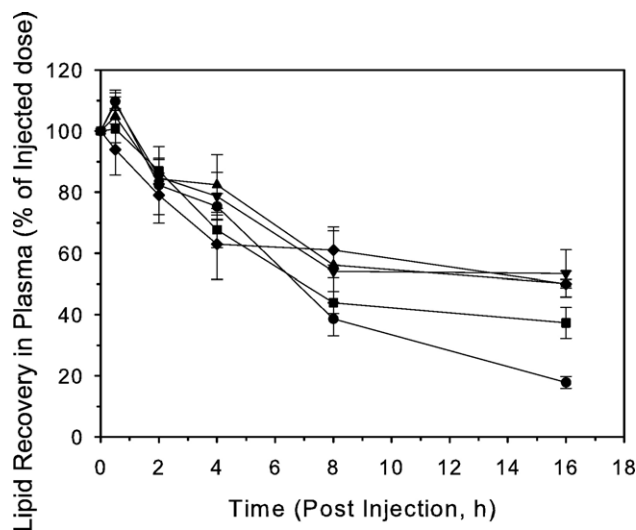


Fig. 5. Plasma lipid recovery (% of injected dose) of SM/Chol (55/45, mol) liposomes in vivo. ICR mice were injected intravenously with SM/Chol formulations of vincristine having drug-to-lipid ratios (wt/wt) 0.025 (∇), 0.05 (\blacktriangle), 0.1 (\blacksquare), 0.33 (\bullet) and 0.4 (\blacklozenge). All mice received lipid doses at 40 mg/kg total lipid and vincristine at 2 mg/kg drug, with the exception of the 0.025 D/L ratio sample which was dosed at 1 mg/kg vincristine and the 0.4 D/L ratio sample which was dosed at 20 mg/kg vincristine and 50 mg/kg total lipid. Data points represent mean values (\pm standard deviations) calculated from 3 mice.

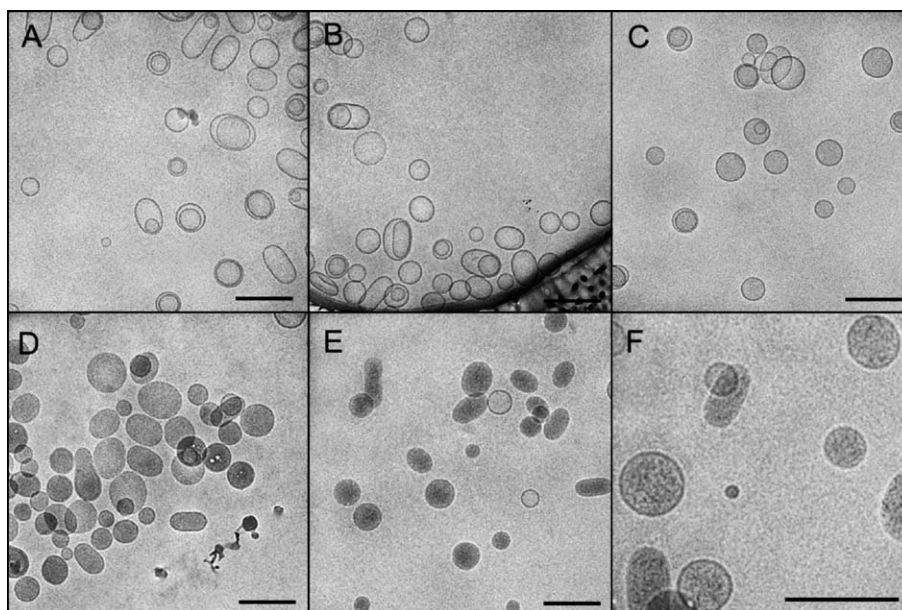


Fig. 6. Cryo-transmission electron microscopy of SM/Chol (55/45, mol) liposomes containing vincristine at different drug-to-lipid ratios. Liposomes containing 300 nm internal magnesium were loaded with vincristine using the ionophore method as described in Materials and methods. Panels represent empty liposomes (A), and *D/L* ratios (wt/wt) of 0.06 (B), 0.27 (C), 0.6 (D) and 1.03 (E). Panel F represents an enlarged section of Panel E and shows granulated structures within the liposomes at higher *D/L* ratios. The bar in panel A represents 200 nm and all micrographs (A–E) are shown at the same magnification. Each panel is a representative image taken from at least 5 images per *D/L* ratio.

evidence that vincristine can form internal precipitates when loaded into LUVs.

3.5. A therapeutically optimized drug release rate can be achieved for liposomal vincristine

Early studies on liposomal vincristine demonstrated that the anti-tumor efficacy in a variety of tumor models was very sensitive to the drug release rate. In particular, the efficacy of liposomal vincristine in the L1210 [8], P388 [33,40] and A431 [21] tumor models was significantly improved over free drug and showed further improvement as the rate of release of vincristine was decreased. Obviously, if the rate of drug release is sufficiently slow, reduced activity would be expected, however such slow release rates were not achievable with the technology then available. The formulations with the slowest rates of release consisted of SM/Chol (55:45) loaded at a *D/L* ratio of 0.1 in response to a pH gradient established by entrapped phosphate buffer, which demonstrated a $T_{1/2}$ for drug release of approximately 24 h *in vivo* [21]. This formulation is currently in advanced clinical trial for treatment of non-Hodgkin's lymphoma [41].

In order to demonstrate that a therapeutically optimized drug release rate does exist, at least for one tumor model, the antitumor efficacy of liposomal vincristine was evaluated in the MX-1 human breast cancer model employing formulations with a variety of *D/L* ratios and corresponding large variations in the rates of drug release. Formulations with *D/L* ratios of 0.025 ($T_{1/2}$ = 6.1 h), 0.05 ($T_{1/2}$ = 8.7 h), 0.1 ($T_{1/2}$ = 15.6 h) and 0.6 ($T_{1/2}$ = 117 h, as extrapolated from the results of Fig. 4b), were employed with the hope of bracketing the optimum release rate.

Tumors were induced in the flanks of female animals, allowed to grow for 14 days (200–300 mm³) and then the mice were treated with a single intravenous dose of liposomal vincristine (1.5 mg/kg) using empty liposomes to maintain a constant lipid dose as the *D/L* ratio was increased. All treatment

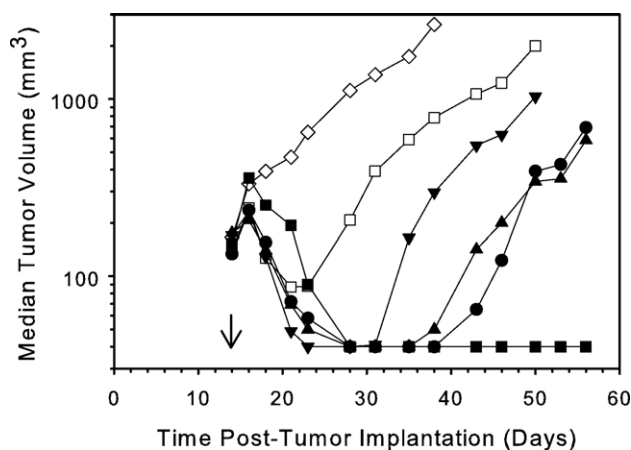


Fig. 7. Therapeutic effects of liposomal vincristine formulations with different *D/L* ratios (and correspondingly different drug release rates) in the MX-1 human mammary xenograft model. MX-1 tumors were implanted in the dorsal flank of nude mice as described in Materials and methods. Once tumors were appropriately sized (~150 mm³), mice were treated with 300 nm sucrose (◇), 1.5 mg/kg free vincristine alone (□), or various formulations of liposomal vincristine (SM/Chol, 55/45, mol) at a dose level of 1.5 mg/kg vincristine and 66 mg/kg total lipid, with *D/L* ratios (wt/wt) of 0.025 (▼, $T_{1/2}$ = 6.1 h), 0.05 (▲, $T_{1/2}$ = 8.7 h), 0.1 (■, $T_{1/2}$ = 15.6 h) and 0.6 (●, extrapolated $T_{1/2}$ = 117 h). Empty SM/Chol liposomes were used at the higher *D/L* ratios to keep the total lipid dose at 66 mg/kg. All mice began treatment on day 14 (arrow), median tumor volumes are displayed (n = 5).

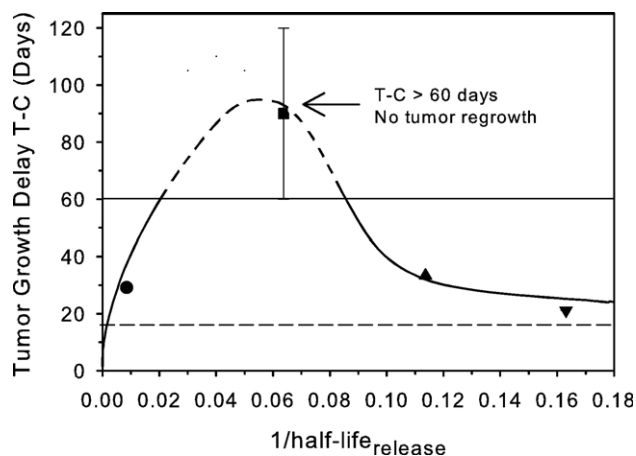


Fig. 8. Therapeutically optimized rates of drug release for liposomal vincristine in MX-1 human mammary xenograft model are in the range of 0.067 h^{-1} ($T_{1/2} = 15.6 \text{ h}$). This figure depicts tumor growth delay ($T-C$, the time taken for tumors in the treatment group to reach a tumor volume of 1000 mm^3 minus the time taken for the control group) as calculated from the tumor growth profiles observed in Fig. 7, as a function of the rate of vincristine release ($T_{1/2}^{-1}$). The symbols represent $T-C$ for each of the different D/L ratio (wt/wt) formulations: 0.025 (▼), 0.05 (▲), 0.1 (■) and 0.6 (●). The dashed line represents the tumor growth delay for a single intravenous dose of vincristine at 1.5 mg/kg . Note that for the point corresponding to a drug release rate of 0.067 h^{-1} $T-C$ is much greater than 60 days as there was no tumor re-growth at this release rate. For didactic purposes the $T-C$ has been arbitrarily set at 90 days for this release rate.

groups demonstrated reversible weight loss compared with untreated animals, and mice that received the high D/L ratio formulations, which displayed progressively slower drug release rates, generally exhibited less weight loss (data not shown). All treatment groups, including vincristine alone, showed initial tumor regressions at the vincristine dose used (1.5 mg/kg), followed by tumor regrowth in most groups. As shown in Fig. 7, in all cases, liposomal vincristine showed superior efficacy over vincristine alone, regardless of the D/L ratio. Further, significant differences in efficacy between liposomal formulations were observed depending on the D/L ratio used. The tumor growth delay $T-C$, which is the time taken for tumors in the treatment group to reach a tumor volume of 1000 mm^3 minus the time taken for the control group, is a commonly used parameter to measure relative efficacy of different formulations. As noted in Fig. 8, $T-C$ values increased from 21 days at the lowest D/L ratio (0.025, wt/wt, $T_{1/2} = 6.1 \text{ h}$) to greater than 60 days at a 0.1 D/L ratio ($T_{1/2} = 15.6 \text{ h}$), at which time all animals were tumor free. Importantly, further increases in the D/L ratio up to 0.6 (wt/wt) ($T_{1/2} \text{ release} = 117 \text{ h}$), resulted in a decrease in activity ($T-C = 29$ days). At this D/L ratio, drug retention is long and the reduced activity observed suggests that drug release is sufficiently slow that antitumor activity is compromised.

4. Discussion

This study demonstrates that the release rates of vincristine from liposomes can be regulated by varying the drug-to-lipid ratio and that, at high D/L ratios in SM/Chol liposomes, therapeutically optimized rates of release can be achieved. There are four major points of interest. The first concerns the

extremely high D/L ratios that are demonstrated here for vincristine, which are considerably higher than achieved previously. The second concerns the mechanism whereby changes in the D/L ratio can modulate drug release rates. The third concerns the great variations in activity that are observed for liposomal formulations of vincristine with different release rates and how this may relate to the drug mechanism of action. A final point concerns whether optimized release rates determined in specific cancer models are relevant to liposomal formulations used clinically.

The results reported here demonstrate that vincristine can be loaded into LUVs, employing the ionophore loading procedure, to achieve D/L ratios as high as one, which is close to the maximum that is theoretically possible (the vincristine levels that could theoretically be achieved in exchange for 300 mM Mg^{2+} entrapped in a 100-nm diameter LUVs corresponds to a D/L ratio of ~ 1.1). Such high D/L ratios have not been reported previously for any drug or drug loading protocol. For example, the highest levels of doxorubicin reported [42,43] correspond to D/L ratios of approximately 0.3 (wt/wt), for vincristine to 0.3 (wt/wt) [27], for topotecan to 0.3 (wt/wt) [38] and vinorelbine to 0.3 (wt/wt) [44]. Aside from the obvious utility of systems with very high D/L ratios for achieving extremely slow release times in vivo (the $T_{1/2}$ for a liposomal vincristine system with a D/L ratio of one can be extrapolated to be approximately 8 days in vivo), these systems are of interest because of the extremely high potency of each liposomal entity. For example, a D/L ratio of one corresponds to approximately 84,000 molecules of vincristine per 100 nm diameter liposome (see Appendix A). Although it is difficult to accurately determine the number of vincristine molecules required to kill a cell, an estimate can be achieved from the IC_{50} values (concentrations of drug required to inhibit cell growth in vitro by 50%) that have been reported in the literature. In particular, for exposure times of 24–48 h, IC_{50} values in the range of 10 nM have been observed for L1210 murine leukemia cells [8] and for a variety of human tumor cell lines [45]. These studies employed approximately 10^5 cells per well. Although neither of these studies reported the actual volume of 10 nM vincristine that was added to the wells, a volume of $200 \mu\text{l}$ would be a reasonable assumption. It may then be calculated that the IC_{50} corresponds to approximately 10^7 molecules of vincristine per cell. Thus, even in the unlikely event that all of the vincristine in the solution partitions into the cells and is required to inhibit cell growth, this would be supplied by only 120 liposomes with a D/L ratio of 1. In the more realistic scenario, where the drug partitions into the cells according to its octanol–water partition coefficient ($\log P = 2.14$) [46], the concentration in the cells would be approximately $1.4 \mu\text{M}$, corresponding to approximately 440,000 vincristine molecules per $10 \mu\text{m}$ diameter cell, requiring delivery of only five liposomes per cell. Such highly potent liposomes may well be of utility in targeting applications, particularly when the number of cell surface receptors that are targeted is limited.

The mechanism whereby higher D/L ratios lead to reduced release rates is of obvious interest. As indicated under Results, if drug release is governed by the usual Fick's law relationship, the efflux rates should be proportional to the total amount of encapsulated drug and the percent of drug released over time should be independent of the initial interior drug concentration,

which is inconsistent with the observed behavior. The results can, however, be accounted for if the large bulk of the encapsulated drug exists in a precipitated form at equilibrium with a small proportion of the drug in the soluble form. Under these conditions the efflux rate will be proportional to the concentration of the soluble form of the drug, which will remain constant until the precipitated form is dissolved. Thus, the half-time ($T_{1/2}$) for drug release will be proportional to the amount of drug in the precipitated form. If the bulk of the drug is in the precipitated form over the range of D/L ratios examined, the half-times for drug release from the various formulations will then be directly proportional to the initial D/L ratio, as observed experimentally.

The proposal that vincristine precipitates in the liposome interior is consistent with the results presented here, particularly the cryo-EM studies on formulations with the highest D/L ratios, where well defined interior structures became apparent at D/L ratios of 1. At lower D/L ratios the liposome interior becomes increasingly electron dense, however precipitated particulates are difficult to discern. It is likely that the increasing electron density reflects the presence of precipitated vincristine in an amorphous form, given the linear dependence of the release rate on the D/L ratio over the entire range of D/L ratios examined. The appearance of the precipitate contrasts with that observed for liposomal formulations of other anti-cancer drugs such as doxorubicin [47], mitoxantrone [48] and topotecan [38], where well-defined internalized precipitates are observed at much lower D/L ratios. In the case of topotecan [49] it was noted that a formulation with a D/L ratio of 0.2 exhibited internal precipitates and improved retention compared to formulations with lower D/L ratios, consistent with the results presented here for vincristine.

It has long been recognized that the efficacy of liposomal formulations of vincristine is highly sensitive to the drug release rate, a point that is reinforced by the results presented here employing the MX-1 human breast cancer model. Early studies using the L1210 and P388 murine leukemia models [16,40], and the A431 human squamous carcinoma model [21] demonstrated that formulations with slow release rates essentially converted the drug from being inactive to one that produced 100% cures. This behavior contrasts with that of other drugs, such as doxorubicin, where release rates have a much smaller effect on activity in the L1210 model [43] or the orthotopic 4T1 murine mammary carcinoma model [50]. In the latter study, the maximum tumor growth delays were less than 50% even though the doxorubicin halftimes for release were varied over a range of approximately 7 h to 10 days. It has been proposed [51] that the reason for this difference concerns the fact that vincristine is a cell cycle-specific drug [52,53], whereas doxorubicin is not [54]. Extended drug release at tumor sites would be expected to have the greatest benefit for cell cycle-specific agents, such as vincristine, where strong relationships between tumor cell exposure times and enhanced cell killing have been established [10–15].

The sensitivity of the activity of liposomal formulations of vincristine to the release rate of the drug naturally leads to the question of what is the therapeutically optimized rate of drug

release. As indicated in Fig. 8, the efficacy of formulations with different payout rates would be expected to exhibit little or no activity if the drug is released extremely slowly and activity similar to that observed for the free drug if the drug is released very quickly after administration. The fact that at rates of release intermediate between these two extremes enhanced activity compared to the free drug is observed implies that a therapeutically optimized rate of drug release exists. As indicated under Results, the efficacy of previous formulations of liposomal vincristine improved as the rate of drug release was decreased, however rates of drug release that were so slow that activity was compromised could not be achieved and thus truly optimized rates of drug release could not be identified. As demonstrated here, a formulation with a D/L ratio of 0.6 (wt/wt) with an extrapolated *in vivo* $T_{1/2}$ of 117 h exhibits reduced efficacy compared to a formulation with a D/L ratio of 0.1 (wt/wt) with a $T_{1/2}$ of 15.6 h, implying that the optimized rate of drug release corresponds to a $T_{1/2}$ intermediate between 16 h and 117 h. It may be concluded that a formulation with a D/L ratio between 0.1 and 0.6 will exhibit therapeutically optimized properties in the MX-1 human xenograft tumor model.

The final point of discussion concerns the relation between optimized rates of release observed in a human xenograft model and formulations with release rates that are clinically optimal. Human tumors grow over a period of months to years, whereas murine and human tumor xenograft models in mice grow over days, weeks or months. Cell growth rates and tumor doubling times of the various preclinical models would be expected to influence the determination of optimal drug release rates. It is expected that determination of optimal release rates in tumor models will, however, have utility. For example, it is likely that similar levels of free drug are required to achieve tumor cell killing both in xenograft models and in humans. The use of animal models to determine the slowest release rates compatible with activity may therefore be of direct relevance to the clinical situation, provided that similar levels of total (encapsulated) drug are delivered to the tumor site in both the model and in humans. Future work will focus on the influence tumor cell growth rates and repeat dosing schedules on release rates leading to optimized therapeutic properties.

In summary, the results presented here show that vincristine can be loaded into liposomes to achieve very high D/L values, that by varying the D/L values the rates of drug release from the liposomes can be varied over a wide range, that this behavior is consistent with drug precipitation in the LUV interior and that by varying the D/L ratio formulations with therapeutically optimized rates of drug release can be developed. It is expected that such procedures will be applicable to the development of therapeutically optimized liposomal formulations of other cell cycle-specific drugs.

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Appendix A

It is straightforward to show that the total number of vincristine molecules in an SM/Cholesterol liposome is given by the relation

$$N_v = \frac{8\pi R^2(M_s/M_v + M_c/L_r M_v)W_r}{A_s(1 + A_c/L_r A_s)}$$

where: N_v = no. of vincristine molecules per liposome; R = radius of liposome; M_s = molecular weight of sphingomyelin; M_v = molecular weight of vincristine; M_c = molecular weight of cholesterol; L_r = mole ratio of sphingomyelin to cholesterol; W_r = weight ratio of vincristine to lipid; A_s = area per molecule of sphingomyelin; A_c = area per molecule of cholesterol.

Using $R = 50$ nm, $M_s = 703$ [55], $M_v = 824$ [56], $M_c = 387$ [55], $L_r = 55/45$, $A_s = 0.6$ nm² [57], $A_c = 0.4$ nm² [58] then for $W_r = 1$ $N_v = 83,700$.

References

- [1] M. Grunau, 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.
- [2] J.A. Balazsovits, L.D. Mayer, M.B. Bally, P.R. Cullis, M. McDonnell, 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.
- [3] H.J. Lim, D. Masin, T.D. Madden, M.B. Bally, Influence of drug release characteristics on the therapeutic activity of liposomal mitoxantrone, *J. Pharmacol. Exp. Ther.* 281 (1997) 566–573.
- [4] D. Sadava, A. Coleman, S.E. Kane, Liposomal daunorubicin overcomes drug resistance in human breast, ovarian and lung carcinoma cells, *J. Liposome Res.* 12 (2002) 301–309.
- [5] 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.
- [6] 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.
- [7] 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.
- [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. Webb, T. Harasym, D. Masin, M. Bally, L. 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] M.S. Georgiadis, E.K. Russell, A.F. Gazdar, B.E. Johnson, Paclitaxel cytotoxicity against human lung cancer cell lines increases with prolonged exposure durations, *Clin. Cancer Res.* 3 (1997) 449–454.
- [11] H.A. Burris III, A.R. Hanauske, R.K. Johnson, M.H. Marshall, J.G. Kuhn, S.G. Hilsenbeck, D.D. Von Hoff, Activity of topotecan, a new topoisomerase I inhibitor, against human tumor colony-forming units in vitro, *J. Natl. Cancer Inst.* 84 (1992) 1816–1820.
- [12] D.V. Jackson Jr., R.A. Bender, Cytotoxic thresholds of vincristine in a murine and a human leukemia cell line in vitro, *Cancer Res.* 39 (1979) 4346–4349.
- [13] W.R. Bruce, B.E. Meeker, W.E. Powers, F.A. Valeriote, Comparison of the dose- and timesurvival curves for normal hematopoietic and lymphoma colony-forming cells exposed to vinblastine, vincristine, arabinosylcytosine, and amethopterin, *J. Natl. Cancer Inst.* 42 (1969) 1015–1025.
- [14] J.K. Horton, P.J. Houghton, J.A. Houghton, Relationships between tumor responsiveness, vincristine pharmacokinetics and arrest of mitosis in human tumor xenografts, *Biochem. Pharmacol.* 37 (1988) 3995–4000.
- [15] K. Gomi, H. Ohno, K. Nomura, M. Okabe, K. Kobayashi, H. Niitani, Kinetic analysis of combination effect of navelbine (KW-2307) with cisplatin against human lung adenocarcinoma PC-12 cells in culture, *Jpn. J. Cancer Res.* 83 (1992) 532–539.
- [16] 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.
- [17] 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.
- [18] 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.
- [19] 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.
- [20] S. Zellmer, G. Cevc, Tumor targeting in vivo by means of thermolabile fusogenic liposomes, *J. Drug Target.* 4 (1996) 19–29.
- [21] 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.
- [22] L.D. Mayer, R. Nayar, R.L. Thies, N.L. Boman, P.R. Cullis, M.B. Bally, Identification of vesicle properties that enhance the antitumour activity of liposomal vincristine against murine L1210 leukemia, *Cancer Chemother. Pharmacol.* 33 (1993) 17–24.
- [23] N.L. Boman, V.A. Tron, M.B. Bally, P.R. Cullis, Vincristine-induced dermal toxicity is significantly reduced when the drug is given in liposomes, *Cancer Chemother. Pharmacol.* 37 (1996) 351–355.
- [24] A.H. Sarris, F. Hagemester, J. Romaguera, M.A. Rodriguez, P. McLaughlin, A.M. Tsimberidou, L.J. Medeiros, B. Samuels, O. Pate, M. Oholendt, H. Kantarjian, C. Burge, F. Cabanillas, Liposomal vincristine in relapsed non-Hodgkin's lymphomas: early results of an ongoing phase II trial, *Ann. Oncol.* 11 (2000) 69–72.
- [25] F. Guthlein, A.M. Burger, M. Brandl, H.H. Fiebig, R. Schubert, C. Unger, U. Massing, Pharmacokinetics and antitumor activity of vincristine entrapped in vesicular phospholipid gels, *Anticancer Drugs* 13 (2002) 797–805.
- [26] 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.
- [27] I.V. Zhigaltsev, N. Maurer, Q.F. Akhiong, R. Leone, E. Leng, J. Wang, S.C. Semple, P.R. Cullis, Liposome-encapsulated vincristine, vinblastine and vinorelbine: a comparative study of drug loading and retention, *J. Control. Release* 104 (2005) 103–111.
- [28] 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.
- [29] 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.
- [30] M.B.B.M.J. Hope, 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.
- [31] C. Fiske, Y. Subbarow, The colorimetric determination of phosphorus, *J. Biol. Chem.* 66 (1925) 375–400.
- [32] 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.
- [33] 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.
- [34] H.-H. Fiebig, A.M. Burger, in: B.A. Teicher (Ed.), *Tumor Models in Cancer Research*, Humana Press, Totowa, 2002, pp. 113–137.
- [35] 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.
- [36] M.S. Webb, P. Logan, P.M. Kanter, G. St-Onge, K. Gelmon, T. Harasym, L.D. Mayer, M.B. Bally, Preclinical pharmacology, toxicology and efficacy of sphingomyelin/cholesterol liposomal vincristine for therapeutic treatment of cancer, *Cancer Chemother. Pharmacol.* 42 (1998) 461–470.
- [37] G. Haran, R. Cohen, L.K. Bar, Y. Barenholz, Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases, *Biochim. Biophys. Acta* 1151 (1993) 201–215.
- [38] 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.
- [39] D.D. Lasic, B. Ceh, M.C. Stuart, L. Guo, P.M. Frederik, Y. Barenholz, Transmembrane gradient driven phase transitions within vesicles: lessons for drug delivery, *Biochim. Biophys. Acta* 1239 (1995) 145–156.
- [40] N.L. Boman, D. Masin, L.D. Mayer, P.R. Cullis, M.B. Bally, Liposomal vincristine which exhibits increased drug retention and increased circulation longevity cures mice bearing P388 tumors, *Cancer Res.* 54 (1994) 2830–2833.
- [41] J.N. Winter, American Society of Hematology 45th Annual Meeting, American Society of Hematology, San Diego, 2005.
- [42] L.D. Mayer, L.C. Tai, M.B. Bally, G.N. Mitilenes, R.S. Ginsberg, P.R. Cullis, Characterization of liposomal systems containing doxorubicin entrapped in response to pH gradients, *Biochim. Biophys. Acta* 1025 (1990) 143–151.
- [43] L.D. Mayer, L.C.L. Tai, D.S.C. 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.
- [44] 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.
- [45] A. Kruczynski, J.M. Barret, C. Etievant, F. Colpaert, J. Fahy, B.T. Hill, Antimitotic and tubulin-interacting properties of vinflunine, a novel fluorinated Vinca alkaloid, *Biochem. Pharmacol.* 55 (1998) 635–648.
- [46] N.H. Greig, T.T. Soncrant, H.U. Shetty, S. Momma, Q.R. Smith, S.I. Rapoport, Brain uptake and anticancer activities of vincristine and vinblastine are restricted by their low cerebrovascular permeability and binding to plasma constituents in rat, *Cancer Chemother. Pharmacol.* 26 (1990) 263–268.
- [47] X. Li, D.J. Hirsh, D. Cabral-Lilly, A. Zirkel, S.M. Gruner, A.S. Janoff, W.R. Perkins, Doxorubicin physical state in solution and inside liposomes loaded via a pH gradient, *Biochim. Biophys. Acta* 1415 (1998) 23–40.
- [48] M. Almgren, K. Edwards, G. Karlsson, Cryo transmission electron microscopy of liposomes and related structures, *Colloids Surf., A Physicochem. Eng. Asp.* 174 (2000) 3–21.
- [49] S.A. Abraham, C. McKenzie, D. Masin, R. Ng, T.O. Harasym, L.D. Mayer, M.B. Bally, In vitro and in vivo characterization of doxorubicin and vincristine coencapsulated within liposomes through use of transition metal ion complexation and pH gradient loading, *Clin. Cancer Res.* 10 (2004) 728–738.
- [50] G.J. Charrois, T.M. Allen, Drug release rate influences the pharmacokinetics, biodistribution, therapeutic activity, and toxicity of pegylated liposomal doxorubicin formulations in murine breast cancer, *Biochim. Biophys. Acta* 1663 (2004) 167–177.
- [51] 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.
- [52] M.A. Jordan, D. Thrower, L. Wilson, Mechanism of inhibition of cell proliferation by Vinca alkaloids, *Cancer Res.* 51 (1991) 2212–2222.
- [53] R. van Der Heijden, D.I. Jacobs, W. Snoeijer, D. Hallard, R. Verpoorte, The Catharanthus alkaloids: pharmacognosy and biotechnology, *Curr. Med. Chem.* 11 (2004) 607–628.
- [54] S.N. Gardner, A mechanistic, predictive model of dose–response curves for cell cycle phase-specific and -nonspecific drugs, *Cancer Res.* 60 (2000) 1417–1425.
- [55] I. Avanti Polar Lipids, *Products Catalog Edition VI* (1995).
- [56] Sigma, *Biochemicals and Reagents for Life Science Research* (2005).
- [57] G.G. Shipley, L.S. AVECILLA, D.M. Small, Phase behavior and structure of aqueous dispersions of sphingomyelin, *J. Lipid Res.* 15 (1974) 124–131.
- [58] I.V. Grechishnikova, F. Bergstrom, L.B. Johansson, R.E. Brown, J.G. Molotkovsky, New fluorescent cholesterol analogs as membrane probes, *Biochim. Biophys. Acta* 1420 (1999) 189–202.