Intravenous Pretreatment with Empty pH Gradient Liposomes Alters the Pharmacokinetics and Toxicity of Doxorubicin through In Vivo Active Drug Encapsulation

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Abstract ☐ Liposomes have been used widely to improve the therapeutic activity of pharmaceutical agents. The traditional approach for such applications has been to formulate the pharmaceutical agent in liposomes prior to administration in vivo. In this report we demonstrate that liposomes exhibiting a transmembrane pH gradient injected intravenously (iv) can actively encapsulate doxorubicin in the circulation after iv administration of free drug. Small (110 nm) liposomes composed of phosphatidylcholine (PC)/cholesterol (Chol, 55:45 mol:mol) exhibiting a pH gradient (inside acidic) were administered iv 1 h prior to free doxorubicin, and plasma drug levels as well as toxicity and efficacy were evaluated. Predosing with egg PC/Chol pH gradient liposomes increased the plasma concentration of doxorubicin as much as 200-fold compared to free drug alone as well as to predosing with dipalmitoyl PC/Chol pH gradient liposomes or EPC/Chol liposomes without a pH gradient. The ability of the liposomes to alter the pharmacokinetics of doxorubicin was dependent on the presence of a transmembrane pH gradient and correlated with the extent of doxorubicin uptake into the liposomes at 37 °C in pH 7.5 buffer, indicating that doxorubicin was being actively accumulated in the circulating liposomes. This in vivo drug loading was achieved over a range of doxorubicin doses (5 mg/kg–40 mg/kg) and was dependent on the dose of EPC/Chol liposomes administered prior to free doxorubicin injection. The altered pharmacokinetic properties of doxorubicin associated with in vivo doxorubicin encapsulation were accompanied by a decrease in drug toxicity and maintained antitumor potency. These results suggest that pretreatment with empty liposomes exhibiting a pH gradient may provide a versatile and straightforward method for enhancing the pharmacological properties of many drugs that can accumulate into such vesicle systems at physiological temperatures.

Introduction

The developmental process for conventional liposomal anticancer drug formulations most typically includes studies where variations in the physical properties of the lipid carriers (size, lipid composition, and drug-to-lipid ratio) are evaluated in order to select the characteristics that provide optimized therapeutic and toxicity behavior. In addition, pharmaceutical criteria such as trapping efficiency, drug retention, and stability must be satisfied in order for these drug delivery systems to be considered clinically viable. The evolution of liposomal systems that could actively accumulate many lipophilic amine drugs in response to transmembrane ion gradients provided an avenue whereby these demands could be met (see refs 1−3 for review). The use of K^+, H^+, and $(NH_4)_2SO_4$ gradients resulted in liposomal anticancer drug formulations that could be loaded with trapping efficiencies approaching 100% at high drug-to-lipid ratios and for a wide variety of lipid compositions.[1−5] The most extensively studied drugs in this regard are the anthracycline anticancer agents doxorubicin and daunorubicin, both of which have obtained market approval in liposomal formulations.

Although transmembrane ion gradient liposomes have addressed many of the problems facing delivery vehicle-based drug formulations, maintaining the necessary physical and chemical stability properties for pharmaceutically relevant time periods (12−24 months) has still presented significant challenges. Retention of chemically intact drug inside the liposomes after the encapsulation procedure has remained the most problematic characteristic for liposomal formulations of doxorubicin and daunorubicin. This has resulted in formulations that either have a relatively limited shelf life,[6] utilize the transmembrane pH gradient to load the drug just prior to use at the hospital pharmacy[7] or have required extensive development of formulations with physical−chemical properties that will maintain the drug inside the liposomes for time periods beyond 1 year.[8] Clearly, any subsequent liposomal formulations of other drugs that use this encapsulation strategy will require significant characterization and development in this area prior to widespread clinical use.

Given that liposomes display low inherent toxicity and can remain intact in the blood stream over days post iv administration,[9−11] it may be postulated that vesicles with appropriate transmembrane ion gradients could accumulate drugs (those known to respond to these gradients in vitro) while circulating in the plasma compartment. If this could be accomplished, then one may predict that the in vivo loaded liposomes would engender pharmacological properties similar to those observed for drug encapsulated into liposomes prior to injection. This approach could significantly improve the versatility of liposome encapsulation applications, since a single formulation of empty ion gradient liposomes could be readily implemented in a variety of therapeutic applications once their inherent toxicity properties were established in initial Phase I clinical trials. In addition, information on the ability of empty ion gradient liposome to accumulate of drugs in vivo may be very useful in identifying potential interactions between systemic liposomal drug formulations and coadministered pharmaceutical agents.

For in vivo drug encapsulation to be pharmacologically effective, the liposomes would need to (1) retain their transmembrane ion gradient for extended times, (2) be constructed of lipid compositions that will allow membrane permeation and uptake of the drug at physiological tem-

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peratures, (3) be present in the plasma at sufficient concentrations to completely sequester the subsequently administered drug without depleting the transmembrane ion gradient, and (4) rapidly accumulate drug in the plasma before distribution into tissues occurs upon iv drug injection. Data from previous studies suggest that small (approximately 100 nm diameter) egg phosphatidylcholine (EPC[1])/cholesterol (Chol) liposomes exhibiting a pH gradient (inside acidic, see references) may be well suited for such applications. Investigations have demonstrated that these liposomes can efficiently accumulate doxorubicin at physiological temperatures in vitro and, at appropriate doses, are retained for extended periods of time in the circulation.[2,9,12]

In the studies described here, we have evaluated the ability of EPC/Chol liposomes circulating in the plasma compartment to encapsulate doxorubicin administered iv in free form. The selection of doxorubicin for this investigation was based on its well characterized response to liposomes exhibiting a pH gradient as well as the extensive data available on the pharmacological properties of an EPC/Chol liposomal doxorubicin formulation in which the drug is encapsulated prior to in vivo administration.[2,9,12–14] These investigations demonstrate that empty pH gradient liposomes circulating in the central blood compartment can efficiently encapsulate subsequently injected free doxorubicin in a manner that provides improved in vivo activity comparable to that obtained for formulations where the drug is entrapped inside the liposomes prior to administration. The implications of this phenomenon with respect to its utility in favorably altering the pharmacology of drugs as well as potential drug–drug interactions that could arise from a wide range of therapeutic agents is discussed.

Materials and Methods

Materials—Egg PC and DPPC were purchased from Avanti Polar Lipids (Alabaster, AL) while cholesterol and all salts were obtained from Sigma Chemicals (St. Louis, MO). Adriamycin RDF was was obtained from Adria Laboratories (Mississauga, Ontario). Female DBA/2J mice were purchased from Jackson Laboratories (Bar Harbor, MA). Tritiated cholesteryl hexadecyl ether, a nonexchangeable, nonmetabolizable lipid used as a tracer for the liposomes, and ^{14}C-methylamine were purchased from NEN-Dupont (Mississauga, Ontario).

Production of Liposomes—Egg PC/Chol and DPPC/Chol liposomes were produced by lipid thin film hydration/extrusion methods described in detail previously.[15] Briefly, lipid mixtures consisting of EPC and cholesterol (55:45, mol %) were dissolved in $CHCl_3$ and subsequently concentrated to a homogeneous lipid film under a stream of nitrogen gas. The lipid film was placed under high vacuum for at least 4 h prior to hydration at room temperature (EPC/Chol) or 45 °C (DPPC/Chol) with 300 mM citrate buffer pH 4.0 to achieve a final lipid concentration of 100 mg/mL. The sample was frozen and thawed five times[16] before extruding 10 times through two stacked 100 nm pore size polycarbonate filters (Poretics) employing an extrusion device (Lipex Biomembranes, Inc., Vancouver, Canada). The resulting liposomes were sized by QELS using a Nicomp 270 submicron particle sizer operating at 632.8 nm. The liposomes exhibited a mean size distribution of approximately 110 nm. Transmembrane pH gradients were generated in the liposomal preparations prior to administration to mice by dialyzing the liposomes against 1000 volumes of 20 mM Hepes, 150 mM NaCl buffer overnight.

Plasma Clearance Studies—Female DBA/2J mice (18–22 g) were given a single bolus lateral tail vein injection of the indicated doses of liposomes. One hour later, free doxorubicin was injected via the tail vein at the indicated doses. At various times after free doxorubicin administration, mice (4 per group) were terminated by CO_2 asphyxiation. Blood was immediately removed by cardiac puncture and collected into an EDTA-coated microtainer tube. The sample was centrifuged at 500*g* in a clinical benchtop centrifuge for 10 min. Plasma was removed and placed into an eppendorf tube prior to analysis of lipid and/or doxorubicin.

Quantitation of Liposomal Lipid and Doxorubicin—Liposomal lipid was quantified by employing the nonexchangeable and nonmetabolizable lipid marker, ^{3}H-cholesteryl hexadecyl ether.[9] Upon animal termination plasma was assayed for lipid content. The samples (200 μL) were added directly to Pico-fluor 40, and radioactivity was determined by liquid scintillation counting.

Doxorubicin was quantified by a modified Bligh and Dyer based extraction assay as previously outlined.[9] Fifty μL of plasma was diluted to 0.8 mL with distilled H_2O. One hundred μL of 10% sodium dodecyl sulfate (SDS) and 100 μL H_2SO_4 (10 mM) were then added. Subsequently, doxorubicin was extracted into an organic phase following addition of 2 mL of chloroform/isopropyl alcohol (1:1, v/v). The mixture was vortexed vigorously and frozen at −20 °C overnight. After thawing and further vortexing, the samples were centrifuged at 1500*g* for 10 min at room temperature, and the organic phase was collected. The fluorescence of this phase was determined employing a Perkin-Elmer LS 50 B Luminescence Spectrometer with an excitation wavelength of 500 nm and emission wavelength of 550 nm. A standard doxorubicin curve was prepared in blank plasma employing an identical extraction procedure. Drug levels were estimated on the basis of doxorubicin fluorescent equivalents.

Sepharose CL4B gel filtration column separations were performed in order to confirm that doxorubicin, present in plasma containing pH gradient liposomes, was encapsulated inside these vesicles. This allowed for differentiation between free doxorubicin, doxorubicin associated with liposomes and doxorubicin associated with plasma proteins. A 1.5 cm × 18.5 cm Sepharose CL4B column was packed using 20 mM Hepes, 150 mM NaCl buffer, pH 7.5. The elution pattern of protein (plasma from untreated mice), free doxorubicin in plasma, doxorubicin encapsulated in EPC/Chol (55:45, mol/mol) liposomes, and liposomes containing doxorubicin was characterized by loading 200 μL of sample onto the column and collecting 0.6 mL fractions using a Gilson Micro Fractionator fraction collector. Protein content was determined using the Sigma Bicinchonic Acid Protein Assay. A protein standard curve was prepared from a stock bovine serum albumin solution. Two mL of protein determination reagent (4% copper(II) sulfate pentahydrate solution: bicinchoninic acid solution; 1:50, v/v) was added to both the standard curve samples and column fraction samples. Samples were incubated at 37 °C for 30 min, and absorbance was measured at 562 nm using a Beckman DU-64 spectrophotometer. Protein levels were determined by regression analysis of the standard curve. Lipid and doxorubicin were quantitated as described above. The gel filtration column was standardized for elution profiles of plasma (protein determination) and 100 nm liposomes as well as doxorubicin in buffer and plasma.

Once these elution patterns had been characterized, CD-1 mice (3/group) were given a 500 mg/kg bolus lateral tail vein injection of EPC/Chol (55:45, mol/mol) liposomes with or without a pH gradient. One hour later, free doxorubicin was injected via the tail vein at 20 mg/kg. One hour after free doxorubicin administration, the mice were terminated by CO_2 asphyxiation. Blood was removed and processed as previously above. Plasma samples from each group were pooled and run down the CL4B column. Fractions were collected and analyzed for lipid and doxorubicin as described above.

Determination of Transmembrane pH Gradient in Liposomes Recovered from Mouse Plasma—At various times after iv injection of pH gradient-bearing liposomes to DBA/2J mice, ^{14}C-methylamine was added to the collected plasma to achieve 0.1 μCi/mL. After 10 min incubations at room temperature, 150 μL samples were applied to 1 mL Sephadex G-50 spin columns[2] followed by 50 μL of Hepes/NaCl pH 7.5 buffer. The liposome-containing eluant fraction was collected, and aliquots from this fraction as well as the precolumn plasma samples were quantified for radioactivity by liquid scintillation counting. The transmembrane pH gradient was then determined by correlating the intravesicular and extravesicular methylamine concentration to transmembrane proton concentration gradients as described previously.[17]

Toxicity and Efficacy Studies—The toxicity of doxorubicin administered to mice injected 1 h previously with empty liposomes was assessed in dose range-finding studies using female DBA/2J mice. Mice were administered increasing doses of doxorubicin until either the weight loss nadir was greater than 20% or any mice died. Mice were monitored twice daily over 14 days for survival and signs of stress (ruffed coat, lethargy, impaired gait, etc.). The

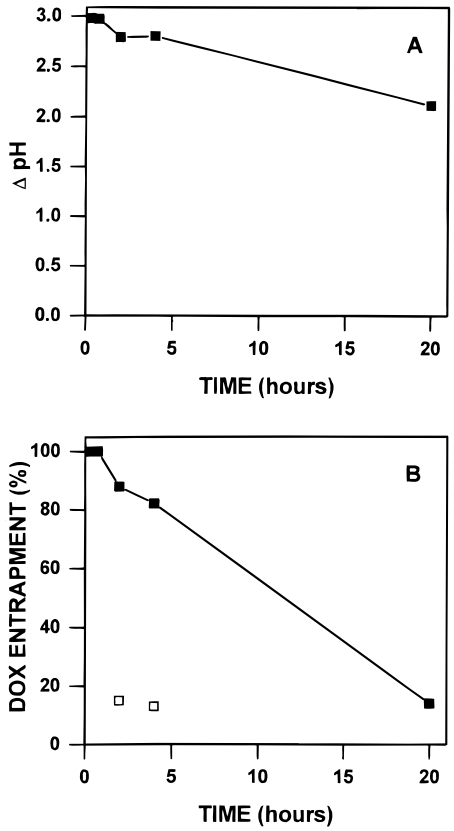

Figure 1—100 nm EPC/CHOL liposomes were administered IV to mice at a dose of 100 mg lipid/kg, and at the indicated times the liposomes recovered in the plasma were tested for residual pH gradient (panel A) and DOX trapping efficiency (panel B). Liposomes were administered exhibiting an internal pH of 3.0 (closed squares) or 7.5 (no pH gradient, open squares).

maximum tolerated dose of doxorubicin was defined as that where no deaths were observed and the body weight loss nadir was ≤15%. The L1210 ascites lymphocytic leukemia was utilized to determine the antitumor activity for the various treatment groups. In this model, 1×10^5 L1210 cells (maintained by serial passage in DBA/2J mice) were injected intraperitoneally in female DBA/2J mice (10/group) and iv doxorubicin treatment was initiated 24 h later (1 h after empty liposome iv injection, where indicated). Mice were then monitored twice daily for survival and weight loss/gain. The extent of antitumor activity was revealed as an increase in the survival time of treated mice relative to untreated mice (saline injections only). Comparisons were based on the percent increase in life span (%ILS) which was calculated as [(median survival time of treated mice ÷ median survival time of control mice) − 1] × 100. Statistical significance of differences between different groups was determined employing a two-tailed Wilcoxon ranking test.

Results

Initial experiments were designed to evaluate the ability of EPC/Chol liposomes to retain their transmembrane pH gradient in the blood compartment after iv injection and subsequently accumulate doxorubicin. These features are minimum criteria that must be met for the liposomes to be capable of altering the pharmacology of doxorubicin through in vivo drug encapsulation. Liposomes exhibiting a 3.5 unit pH gradient (pH 4.0 inside/7.5 outside) were injected iv at a dose of 100 mg total lipid/kg. At various times blood was harvested and the pH gradient of liposomes in the plasma was determined using ^{14}C-methylamine as described in Materials and Methods. As shown in Figure 1A, the EPC/Chol liposomes exhibited a pH gradient between 2.75 and 3.0 units over 4 h post administration. This pH gradient decreased to 2.0 units by 20 h.

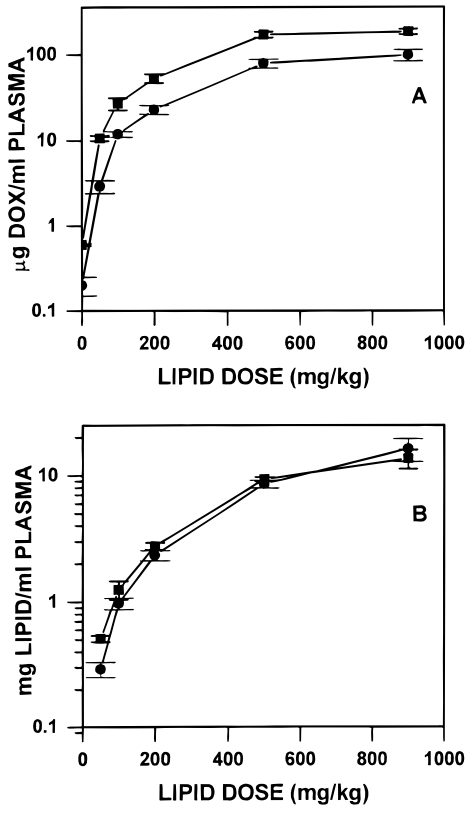

Figure 2—DOX (panel A) and lipid (panel B) concentrations in the plasma at 1 h (■) and 4 h (●) after administration of DOX at 20 mg/kg to mice pretreated with EPC/Chol pH gradient liposomes.

As a control, EPC/Chol liposomes exhibiting no pH gradient (pH 7.5 inside and outside) administered at 100 mg/kg yielded pH gradient measurements of approximately 0.5 units (data not shown). The basis of this small, nonspecific pH gradient appeared to reflect methylamine binding to either protein or lipoprotein components that coeluted with the liposomes during the gel filtration plasma processing step. Quantification of the liposomal lipid remaining in the plasma for pH gradient bearing liposomes at the dose of 100 mg/kg indicated that greater than 72%and 13% of the administered dose of liposomes remained in the circulation at 4 h and 20 h, respectively. Comparable lipid levels were observed for EPC/Chol liposomes in the absence of a pH gradient (data not shown).

Free doxorubicin was added to an aliquot of the liposome-containing plasma samples, and doxorubicin uptake into the vesicles was determined using the same gel filtration procedure as described for methylamine analysis. Liposomes obtained over the first hour after iv injection were able to encapsulate the doxorubicin with trapping efficiencies approaching 100% (Figure 1B). At the 2 h, 4 h, and 20 h timepoints, the efficiency of doxorubicin entrapment fell to 88%, 82%, and 14%, respectively. In contrast, EPC/Chol liposomes without a pH gradient provided doxorubicin entrapment levels that were approximately 10-fold lower than obtained for pH gradient bearing liposomal systems.

The data presented above suggest that optimal in vivo encapsulation of doxorubicin into EPC/Chol vesicles will be obtained when the doxorubicin is injected within 1 h of pH gradient-bearing liposome administration. Using this dosing schedule, the influence of liposome and doxorubicin dose was investigated in order to establish conditions that lead to optimized in vivo drug encapsulation and pharmacokinetic properties. In the first set of experiments, the dose of small (110 nm) EPC/Chol liposomes bearing a 3.5 unit pH gradient was varied from 50 mg/kg to 900 mg/kg. At 1

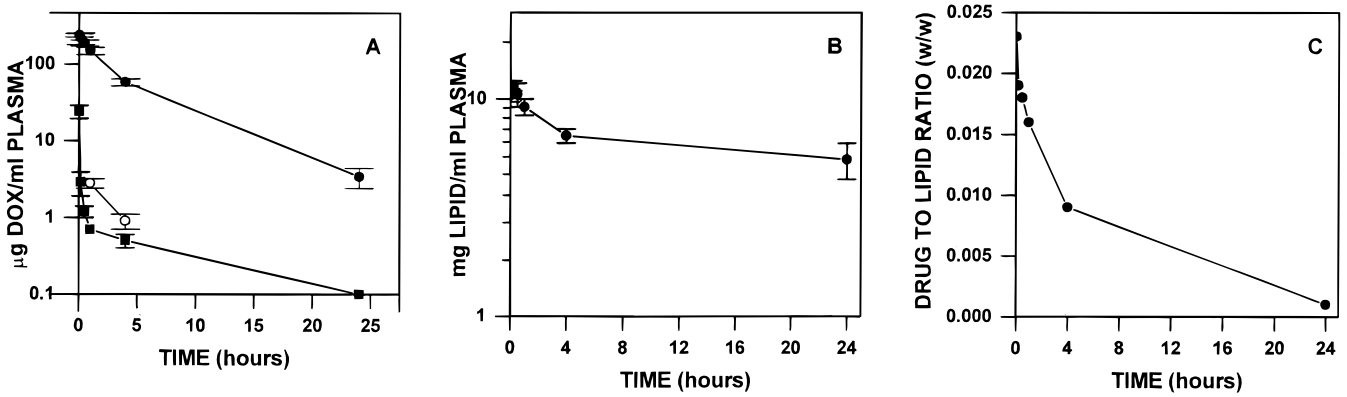

Figure 3—DOX (panel A) and liposomal lipid (panel B) plasma concentrations and circulating drug-to-lipid ratio (panel C) after administration of DOX in free form to mice in the absence (■) and presence (●) of a 500 mg/kg pretreatment dose of 100 nm EPC/CHOL liposomes.

h after iv liposome administration, doxorubicin was injected iv at a dose of 20 mg/kg. At a liposome dose of 50 mg/kg, the concentration of doxorubicin in the plasma was 17.6- and 14.5-fold higher than that observed for free doxorubicin injected in the absence of liposomes at 1 h and 4 h after drug administration, respectively (Figure 2A). Increases in the liposome dose led to increases in the concentration of circulating liposomes as well as the concentration of doxorubicin in plasma. Whereas the concentration of liposomes in the circulation increased proportional to the liposome dose between 50 and 1000 mg/kg for both 1 h and 4 h time points (Figure 2B), the concentration of doxorubicin in the plasma reached plateau levels at a liposome of 500 mg/kg (Figure 2A). At this dose of EPC/Chol pH gradient bearing liposomes, the circulating drug-to-lipid ratios (wt/wt) at 1 h and 4 h after 20 mg/kg doxorubicin injection were 0.02 and 0.01, respectively. Increasing the liposome dose further from 500 mg/kg to 1000 mg/kg resulted in a small increase in circulating doxorubicin concentrations from 170.9 +13.5 μg/mL to 184.8 ± 13.2 μg/mL.

A more detailed pharmacokinetic study was undertaken in order to characterize the effect of in vivo liposome encapsulation on the plasma elimination properties of doxorubicin. Mice were administered pH gradient-bearing EPC/Chol liposomes at a dose of 500 mg/kg 1 h prior to iv injection of 20 mg/kg doxorubicin. Mice were then sacrificed at various times postdrug injection, and their plasma was analyzed for doxorubicin and liposomal lipid as described in Materials and Methods. A comparison was made with doxorubicin injected iv at 20 mg/kg in the absence of any liposome pretreatment. As shown in Figure 3A, doxorubicin in the absence of liposomes is eliminated very rapidly from the circulation after iv injection, with a minimum of two distinct elimination phases. Within 2 min postinjection, >94% of the drug administered has been removed from the plasma and the level of drug eliminated increases to >99.7% within 30 min. In contrast, doxorubicin administered to mice pretreated with 500 mg/kg EPC/Chol pH gradient bearing liposomes is eliminated much more slowly, where <36% and <42% of the drug is removed from the plasma at 2 and 30 min after drug injection, respectively. The increases in doxorubicin concentration observed for mice pretreated with pH gradient liposomes compared to no pretreatment were 9.9-, 157.6-, 115.2-, and 35.0-fold at 2 min, 30 min, 4 h, and 24 h after doxorubicin administration, respectively. The corresponding 0−24 h trapezoidal area under the curve (AUC) values calculated using all time points were 5.5 μgh/mL for doxorubicin alone and 408.8 μgh/mL for doxorubicin in mice pretreated with 500 mg/kg EPC/Chol liposomes which reflected a 74.3-fold increase in total doxorubicin exposure in the plasma. Pretreatment with empty EPC/Chol liposomes prepared without a pH gradient (pH 7.5 inside and outside) at 500 mg/mL resulted in circulating doxorubicin levels that were comparable to those observed with free doxorubicin alone (Figure 3A). In addition, pretreatment with pH gradient-bearing liposomes composed of DPPC/Chol (55:45 mol %) provided circulating doxorubicin concentrations that were <10% of those obtained with EPC/Chol liposomes at 1 h and 4 h after drug administration (data not shown).

Although the plasma doxorubicin concentration in mice pretreated with EPC/Chol liposomes decreased from 237.4 ± 12.3 μg/mL at 2 min postinjection to 3.4 ± 1.5 μg/mL at 24 h (70-fold reduction), the circulating liposomal lipid levels over this time period decreased by only 2-fold from 10.4 ± 0.7 mg/mL to 5.3 ± 1.0 mg/mL (Figure 3B). As a result, the circulating drug-to-lipid ratio for this treatment group decreased from an initial (2 min) value of 0.023:1 (wt/wt) to a value of 0.0006:1 at 24 h (Figure 3C). This drug release (as defined by changes in the drug-to-lipid ratio) followed first-order kinetics and led to a reduction of the circulating drug-to-lipid ratio by 63% within 4 h and 97.4% within 24 h. These results suggest that the circulating liposomes initially accumulate the doxorubicin after it is injected iv and then slowly release the drug subsequently while remaining in the central blood compartment.

The fact that increased doxorubicin concentrations in plasma were obtained only when EPC/Chol liposomes exhibiting a pH gradient (inside acidic) were utilized as a pretreatment before free doxorubicin administration strongly suggested that doxorubicin was being actively sequestered inside the liposomes in response to the pH gradient. This was confirmed by chromatographing plasma from mice treated with EPC/Chol liposomes either with or without a pH gradient (500 mg/kg) and 20 mg/kg free doxorubicin on a Sepharose CL4B column (Figure 4). This resulted in the separation of liposomes (and entrapped doxorubicin) from plasma proteins and free drug. The column was first calibrated for elution profiles of empty liposomes, plasma protein content, and doxorubicin incubated in plasma. Liposomes were readily separated from plasma proteins and free doxorubicin, and it was observed that >90% of doxorubicin in plasma was recovered in the included volume of the column (Figure 4). When plasma obtained 1 h after doxorubicin injection to EPC/Chol liposome (with pH gradient) pretreated mice was applied to this column, >95% of the liposomal lipid and doxorubicin detected was recovered in the liposome fraction. Due to the very low doxorubicin concentrations in plasma after pretreatment with empty EPC/Chol liposomes without a pH gradient, doxorubicin could not be detected in any of the elution fractions (data not shown). It should be noted, however, that lipid concentrations for this treatment group were

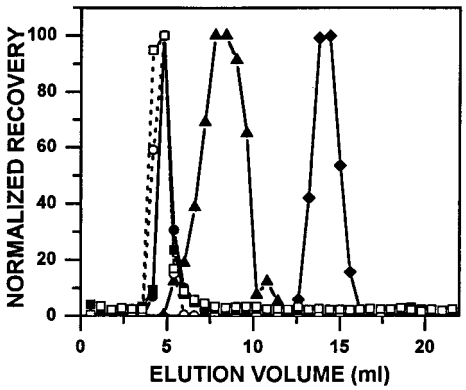

Figure 4—Sepharose CL4B elution profiles for liposomes and doxorubicin incubated in plasma (solid lines) or plasma from mice administered 500 mg/kg pH gradient-bearing EPC/Chol liposomes followed 1 h later by 20 mg/kg free doxorubicin (dashed lines). Elution fractions were analyzed for protein (▲), doxorubicin (◆), liposomal lipid for pre-encapsulated EPC/Chol doxorubicin (●), and doxorubicin pre-encapsulated in EPC/Chol pH gradient liposomes (■) to calibrate the elution positions of the various components. Doxorubicin (○) and liposomal lipid (□) were analyzed from mouse plasma following iv administration of 100 nm EPC/CHOL liposomes containing a pH gradient and free doxorubicin.

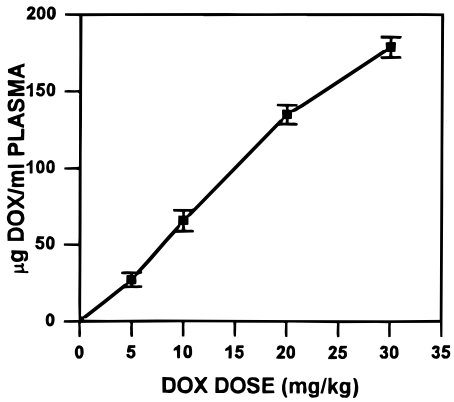

Figure 5—Pretreatment with 100 nm empty pH gradient EPC/CHOL liposomes was administered iv at 500 mg lipid/kg, and plasma DOX concentrations were determined at 4 h after free DOX administration iv at the indicated doses.

identical to those for pH gradient bearing liposomes and all of the liposomal lipid was recovered in the liposome elution volume.

In addition to altering the dose of pH gradient-bearing liposomes, the dose of doxorubicin administered after the liposome pretreatment was manipulated in order to determine the drug–dose dependence of the liposome induced pharmacokinetic changes. As shown in Figure 5, increasing the doxorubicin dose from 5 mg/kg to 30 mg/kg increased the plasma concentration of drug 4 h post iv injection from 27.1 ± 4.6 μg/mL to 178.8 ± 6.6 μg/mL. This increase was proportional to the doxorubicin dose where the percent administered doxorubicin recovered in the plasma at 4 h remained relatively constant (range of 29% to 35%) over a 6-fold range of drug dose. These results indicate that the doxorubicin pharmacokinetic alterations induced by pH gradient-bearing EPC/Chol liposomes are dose independent with respect to doxorubicin over the range studied here.

The biological activity evaluation of the in vivo drug encapsulation strategy described above was performed in order to correlate the changes in doxorubicin pharmacokinetics induced by pH gradient liposome pretreatment with toxicity and efficacy properties. These characteristics were also compared to treatment with free drug alone as well as doxorubicin administered in traditional liposomal formulations where the drug is encapsulated prior to use.

Table 1—Effect of In Vivo Liposomal Capture on Doxorubicin Toxicity[a]

group	DOX dose (mg/kg)	weight loss nadir (%)	toxicity-related mortality
free DOX	20	16	0/6
	25	26	2/6
	30	NA	6/6
preloaded	20	2	0/6
liposomal DOX	30	15	0/6
	40	NA	6/6
in vivo capture	20	5	0/6
liposomal DOX	30	16	0/6
	40	NA	6/6

[a] Preloaded liposomal DOX was prepared by entrapping DOX into 100 nm EPC/Chol liposomes exhibiting a 3.5 unit transmembrane pH gradient (inside acidic). In vivo capture DOX was performed by pretreating DBA-2J mice iv with empty pH gradient bearing EPC/Chol liposomes 1 h before iv injection of free DOX. NA: not applicable since animals died and nadirs cannot be accurately determined.

Table 2—Therapeutic Efficacy of Doxorubicin in Free Form, Preloaded Liposome Encapsulated Form and in Vivo Liposomal Capture Form against L1210 Murine Ascitic Leukemia Model[a]

group	dose (mg/kg) lipid	dose (mg/kg) DOX	median survival time (days)	60-day survival	%ILS
saline control	–	–	11.0	0/6	–
liposomes alone	500	0	10.5	0/6	–5
free DOX	0	10	18.5	1/12	68
	0	20	29.0	1/12	164
preloaded	50	10	19.5	0/6	77
liposomal DOX	100	20	31.0	1/6	182
in vivo capture	500	10	19.0	0/6	73
liposomal DOX	500	20	28.5	1/6	159

[a] Preloaded liposomal DOX was prepared by entrapping DOX into 100 nm EPC/Chol liposomes exhibiting a 3.5 unit transmembrane pH gradient (inside acidic). In vivo capture DOX was performed by pretreating tumor-bearing mice iv with empty pH gradient bearing EPC/Chol liposomes 1 h before iv injection of free DOX.

The results shown in Table 1 demonstrate that free doxorubicin administered iv to mice in the absence of a liposomes pretreatment exhibits signs of drug toxicity at doses of 20 mg/kg and higher. The occurrence of weight loss nadirs in excess of 20% as well as toxicity-related mortality at 25 mg/kg and above indicated a maximum tolerated dose of 20 mg/kg for this treatment group. In comparison, administration of free doxorubicin to mice pretreated with 500 mg/kg EPC/Chol pH gradient liposomes provided minimal weight loss at 20 mg drug/kg and a weight loss nadir of 16% with no mortality over 14 days was observed at 30 mg/kg (Table 1). However, increasing the doxorubicin dose to 40 mg/kg in pretreated mice resulted in 100% mortality. The dose response for doxorubicin toxicity (manifested by weight loss and mortality) observed in the group pretreated with empty pH gradient-bearing liposomes was very comparable to that obtained for doxorubicin entrapped inside 100 nm EPC/Chol liposomes using the pH gradient entrapment procedure just prior to in vivo administration (Table 1).

The antitumor activity of doxorubicin in mice pretreated with pH gradient-bearing EPC/Chol liposomes was evaluated in the murine L1210 ascites tumor model and compared with free doxorubicin alone as well as conventional liposomal doxorubicin encapsulated in pH gradient liposomes prior to injection. The results shown in Table 2 demonstrate that empty liposomes administered iv at a dose of 500 mg lipid/kg did not provide any antitumor activity compared to the saline treated control group. Free doxorubicin administered iv at 10 mg/kg and 20 mg/kg in

the absence of liposome pretreatment increased the median survival time to 18.5 days and 29.0 days, respectively. These survival times reflected increase in life span (ILS) values of 68% and 164% respectively, and one long-term survivor was observed in each treatment group. Administration of doxorubicin as either the EPC/Chol liposomal formulation (prepared prior to use) or as free drug in mice pretreated with EPC/Chol pH gradient-bearing liposomes provided equivalent antitumor activity as free doxorubicin alone. Median survival times for the conventional EPC/Chol liposomal doxorubicin formulation reflected ILS values of 77% and 182% at drug doses of 10 mg/kg and 20 mg/kg while the group receiving pretreatment with empty EPC/Chol pH gradient-bearing liposomes exhibited ILS values of 73% and 159% at 10 mg/kg and 20 mg/kg, respectively (Table 2). One long-term survivor was observed at the 20 mg/kg drug dose for both groups using liposomes. In all treatment groups, the 20 mg/kg drug dose levels were statistically more therapeutically active than 10 mg/kg ($p < 0.05$); however, no statistical significance could be established between groups treated with different formulations for a given doxorubicin dose.

Discussion

The use of liposomal carriers to improve the therapeutic activity of a variety of pharmacological agents in clinical settings has well established this technology as a viable and important approach in the design of pharmaceutical formulations. Early examples of the benefits of liposome encapsulation came primarily from their ability to decrease drug exposure to healthy, susceptible tissues which led to reductions in target organ toxicities.[2,12,18–21] Subsequently, it became apparent that liposomes also exhibit preferential accumulation into disease sites such as tumors and areas of infection/inflammation, thus providing increased selectivity for disease tissue and improved therapeutic index.[22–24] Various combinations of these properties have been utilized in clinically approved liposomal formulations for drugs such as doxorubicin, daunorubicin, and amphotericin B. For all of these liposomal formulations, a key feature that was important in obtaining regulatory approval was the reduced toxicity compared to their free (nonencapsulated) drug counterparts. This buffering of toxicity allows increased doses to be employed without compromising antitumor potency, thus improving the drug's therapeutic index. For the drugs cited above, many of the toxicities to healthy tissues have been associated with early stage drug distribution phases. Consequently, it is believed that the dramatic reduction of free drug exposed to susceptible tissues after administration of the liposomal formulations is largely responsible for the toxicity buffering effects.

The results presented here not only demonstrate that pH gradient bearing EPC/cholesterol liposomes are capable of encapsulating doxorubicin while circulating in the central blood compartment, but that this entrapment process is very efficient in sequestering free doxorubicin before the drug distributes into tissue compartments throughout the body. This is revealed by the fact that at a liposome pretreatment dose of 500 mg/kg, the amount of doxorubicin in the plasma reflects approximately 50% of the administered drug dose. This is comparable to the results observed previously for EPC/cholesterol liposome formulations with pre-encapsulated doxorubicin where approximately 50% of the entrapped drug is released from the liposomes over the first hour after iv injection.[2] It should be noted, however, that the lipid dose required to achieve this effect is 50-fold higher when pretreatment with empty pH gradient EPC/cholesterol liposomes is employed. Nonetheless, it is striking that the accumulation of doxorubicin into liposomes exhibiting a pH gradient can preempt the early phase distribution of free doxorubicin since >98% of free drug is eliminated from the plasma within 5 min of iv administration in the absence of any liposome pretreatment.

In vivo liposome entrapment of doxorubicin was shown here to be dependent on the presence of a transmembrane pH gradient (inside acidic) as well as low phase transition temperature phospholipid compositions. This indicates that the increased plasma doxorubicin concentrations observed in the presence of liposome pretreatment reflect drug that has been actively sequestered inside the liposomes in response to a pH gradient rather than simple passive partitioning of doxorubicin into the bilayer. Such conclusions are based on the fact that high doses of empty EPC/cholesterol liposomes exhibiting no pH gradient (pH 7.5 inside and outside) did not result in elevated plasma doxorubicin concentrations. In addition, the fact that minimal pharmacokinetic alterations were obtained with DPPC/cholesterol liposomes bearing a pH gradient suggests that doxorubicin uptake into such vesicles is relatively slow at 37 °C,[4] and free doxorubicin may be expected to distribute into tissue compartments prior to accumulation into the liposomes under these conditions. It should be pointed out that although the in vivo drug uptake phenomenon described here was accomplished using a pH gradient, alternate transmembrane ion gradients resulting in membrane potentials sufficient to drive drug accumulation could also provide similar effects.[1,4,5] Indeed, the presence of a transmembrane pH gradient in itself generates a membrane potential that would be expected to facilitate doxorubicin uptake.

Pretreatment with empty EPC/cholesterol liposomes that exhibit a pH gradient provides similar buffering of toxicity effects as EPC/cholesterol liposomes in which doxorubicin has been encapsulated prior to administration. This observation suggests that in vivo entrapment may represent a viable alternative to delivering doxorubicin in preencapsulated form. This approach would have clear advantages regarding problems related to chemical stability and liposome retention integrity that are experienced with traditional liposome entrapped formulations. Further, given that several other cancer chemotherapeutic agents such as other anthracyclines and vinca alkaloids[6,7,25–26] as well as drugs from a wide range of therapeutic classes can be efficiently loaded into liposomes displaying a pH gradient,[27] pretreatment with empty liposomes to improve therapeutic activity could be readily applied and evaluated in a variety of pharmaceutical applications. The pH gradient bearing liposomes may also be capable of favorably altering the pharmacokinetic and toxicity properties of combinations of drugs that can be sequestered into liposomes in response to a pH gradient. This is an important feature for many anticancer treatment regimens where combinations including anthracyclines and vinca alkaloids are often utilized. As such, these studies reveal the therapeutic potential of a novel application of pH gradient bearing liposomes.

A more direct and current application of the results obtained here concerns the potential for preloaded liposomes that exhibit transmembrane ion gradients (such as those employed for clinically used formulations of liposomal doxorubicin, daunorubicin, and vincristine) to sequester additional coadministered drugs in the circulation. This could have significant implications, either adverse or beneficial, on the pharmacology of the sequestered agents. Further, in vivo accumulation of additional agents into preloaded liposomes could affect the retention of the primary encapsulated drug (e.g. doxorubicin, daunorubicin, or vincristine) if the transmembrane ion gradient is altered.

This suggests that increased attention should be given to the pharmacology of relevant coadministered drugs when these types of liposome formulations are administered systemically.

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