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THE ROLE OF LIPOSOME COMPOSITION ON THE STABILIZATION OF ANCITABINE

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ABSTRACT

Reduction in the ancitabine hydrolysis rate in an alkaline medium was achieved by liposome encapsulation of stable ancitabine solutions. The degree of success was gauged using stabilization ratios which compare rates of hydrolysis of free and encapsulated ancitabine under identical conditions. Stabilization ratios as high as 22 were observed when the phase transition

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temperature (PTT) values were 16 to 26°C higher than the storage temperature. Liposomes composed of phospholipids with PTT values below storage temperatures were ineffective. Increased liposome permeability was evidenced by rapid, nearly complete, release of encapsulated ancitabine at temperatures above the PTT. The inclusion of cholesterol in the liposomes reduced the maximum stabilization ratios from 22 to 4.

INTRODUCTION

The pH range for a prospective vehicle for a liposome suspension may be limited by the need for physiological compatibility with the site for drug administration and/or by formulation considerations. However, this restricted pH range may be unfavorable for the stability of the drug which is to be encapsulated within the liposomes. The stabilization potential of liposomes, for a drug which is subject to hydrolysis, is a function of their lipid composition (and hence their phase transition temperature) and the storage conditions. The formulator can influence the physical and chemical stability of a drug-liposome dosage form through the choice of lipids or by the addition of excipients such as cholesterol. Temperature can influence the chemical stability of the liposomes and the drug in addition to altering the permeability of the liposomes.

The purpose of this research was to explore the influence of liposome formulation on the ability of liposomes to protect a labile

drug. Temperature and hydroxyl-ion concentration were used to challenge the system.

Ancitabine was chosen as the model compound for several reasons: 1. Contrary to results for indomethacin[1] and p-nitrophenyl acetate[1,2], ancitabine showed no evidence for association with liposomes or for a change in hydrolysis rate in the presence of positively charged, negatively charged or neutral liposomes.[1] 2. Ancitabine can be quantitatively hydrolyzed to cytarabine at a first-order rate in a convenient time period at moderate temperatures.[3] 3. Ancitabine is relatively stable in the pH range 4 to 6, where the rate is pH independent, but is rapidly hydrolyzed under alkaline conditions.[3] 4. At pH values greater than 7, the ancitabine hydrolysis rate is proportional to hydroxide concentration and independent of buffer concentration.[3] 5. The in vivo rate of conversion of ancitabine to cytarabine is predictable from chemical kinetics showing no evidence for metabolic catalysis.[4]

The ability of liposomes to protect ancitabine from hydrolysis was tested by encapsulating an acidic solution of ancitabine and then suspending the liposomes at an alkaline pH as illustrated in Fig. 1. The rate constants for hydrolysis of ancitabine in these suspensions were compared to those in the absence of liposomes. Significant stabilization would be expected if the inward diffusion of hydroxyl ions and the release of ancitabine were effectively restricted by the liposome membrane (inset, Fig. 1).

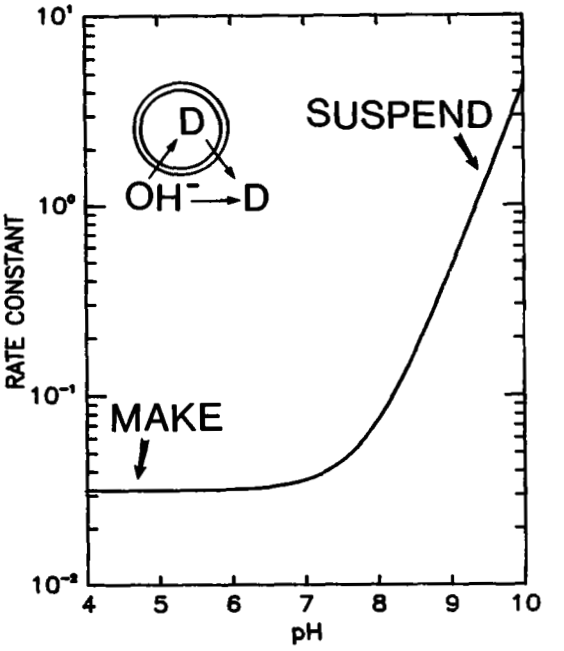

FIGURE 1

Schematic representation illustrating liposomal encapsulation of a drug (D) dissolved in an acidic medium followed by dispersion of the liposomes in an alkaline medium. The curve represents the pH-rate profile for ancitabine at 25° (k in hr^{-1}). The inset shows the processes which would reduce the ability of the liposome to protect the drug from alkaline degradation.

MATERIALS

L-α-phosphatidylcholine (PC), from frozen egg yolk, in chloroform:methanol (9:1) solution; cholesterol (CHOL), and ancitabine were obtained from Sigma Chemical Co. Dimyristoyl phosphatidylcholine (DMPC), dipalmitoyl phosphatidylcholine (DPPC), dipalmitoyl phosphatidylglycerol (DPPG) and distearoyl phosphatidylcholine (DSPC) were obtained from Avanti Polar Lipids and cytarabine was obtained from The Upjohn Co.

METHODS

Preparation of liposomes. Liposomes were prepared by reverse phase evaporation using methods described by Szoka and Papahadjopoulos[5]. The amount of phospholipid (PC, DMPC, DPPC, DSPC) used for liposome formulation was 66 μmoles per mL of acetate buffer. Where specified, either 33 μmoles of cholesterol or 14 μmoles DPPG were also incorporated. The lipid was placed in a 50 mL round bottom flask. When PC was used, the chloroform solvent was removed using a rotary evaporator. Diethyl ether (3 mL) was added to dissolve the lipids. When DPPC or DSPC was employed, 1.5 mL of chloroform was introduced to ensure dissolution prior to addition of ether. One milliliter of aqueous 0.15 M sodium acetate buffer (pH = 4.7) containing 3.8×10^{-2} M (unless otherwise specified) ancitabine was added.

The round bottom flask was flushed with nitrogen during preparation. The mixture was sonicated to form an emulsion (Model W-375; Heat Systems-Ultrasonics Inc.) for 3-5 min at temperatures slightly above the phase transition temperature (PTT) of the phospholipid followed by rotary evaporation at 30°C for PC and DMPC, 41°C for DPPC and 55°C for DSPC. The organic phase was removed by vacuum.

After analyzing the remaining aqueous phase for total ancitabine concentration, the suspension was dialyzed against

100 times its volume of 0.15 M acetate buffer (300 mOsm/L), at 4°C, for 24 h with two changes of dialysis fluid at approximately equal intervals.

In selected studies a 1 mL aliquot of the liposome suspension was diluted with 7 mL of isoosmolar buffer and ultracentrifuged at 41,000 rpm for 15 minutes at 4°C (Beckman L5-50B Ultracentrifuge). The supernatant was analyzed for free ancitabine. The liposome pellet was redispersed in fresh buffer and centrifuged again. Free prodrug was absent after this was repeated three times.

In studies where rapid preparation of liposomes was necessary, unencapsulated ancitabine was removed using ultracentrifugation. The overall time involved to obtain final liposomal preparations using this method was ~1 hour.

The percent ancitabine encapsulated was calculated from

$$\% \text{ encapsulated} = 100\ C_a/C_b \quad (1)$$

where C_b and C_a are the concentrations of ancitabine before and after dialysis and/or centrifugation. Encapsulation was relatively constant at 26-31% except for DPPC:DPPG:CHOL (60%), DSPC:CHOL (46%), and DSPC (10%).

ANALYTICAL. Spectrophotometric (Gilford Model 250 spectrophotometer) analyses of ancitabine and cytarabine in solutions and following encapsulation were carried out as follows.

A 0.3-0.5 mL aliquot of the reaction was added to 5.0 mL of 0.2 M HCl in methanol to quench hydrolysis and dissolve the liposomes. Samples were then stored in the refrigerator. Precipitation occurred on cold storage of DSPC samples but warming restored clear solutions. No loss of ancitabine or cytarabine was observed during storage periods of 48 hours.

The absorbances due to ancitabine and cytarabine were measured at their UV maxima, of 260 and 285 nm respectively. Beer's law plots in 0.2 M HCl in methanol gave molar extinction coefficients of 10.2×10^3 at 260 nm and 3.17×10^3 at 285 nm for ancitabine and 4.28×10^3 and 14.0×10^3 for cytarabine. The total absorbances at the two wavelengths were used to derive

$$10^5 C_1 = 10.8\ A_{260} - 3.31\ A_{285} \quad (2)$$

$$10^5 C_2 = 7.91\ A_{285} - 2.44\ A_{260} \quad (3)$$

from which the molar concentration of ancitabine (C_1) and cytarabine (C_2) in the final dilutions were calculated. The accuracy of the method was tested by determining the concentrations of ancitabine and cytarabine in known mixtures.

Ancitabine hydrolysis kinetics. The suspension of liposomes containing encapsulated ancitabine in acetate buffer (pH 4.7) was diluted with 9 parts of isoosmolar bicarbonate buffer (pH 9.54) and this reaction was maintained at constant temperature. Aliquots were removed as a function of time and analyzed for ancitabine and cytarabine. The flask was flushed with nitrogen

after withdrawing each sample. The hydrolysis in control buffers was studied in the absence of liposomes.

Release of ancitabine from liposomes. The suspension of liposomes containing encapsulated ancitabine was diluted with 9 parts of isoosmolar 0.15 M sodium acetate buffer and maintained at the desired temperature. Aliquots, removed at 5 and 15 minutes, were divided into two portions. One part was assayed for total ancitabine and the other was added to an equal volume of ice cold isoosmolar 0.15 M sodium acetate buffer and centrifuged at 20,000 rpm for 30 minutes. An aliquot of the supernatant was diluted with 0.2 M HCl in methanol and analyzed for ancitabine. The percent released at the end of 5 and 15 minutes was calculated from % released = 100% (C_s/C_t) where C_s is the concentration of ancitabine in the supernatant and C_t is the total concentration in the liposomal suspension.

RESULTS AND DISCUSSION

Ancitabine hydrolysis kinetics. The rate of ancitabine hydrolysis was measured after liposomes containing acidic ancitabine solution (pH ~4.7) were suspended in an alkaline environment (pH ~9.5). Rates were generally described by

$$\ln F = -kt \qquad (6)$$

where F is the ancitabine fraction at time t and k is the observed first-order rate constant denoted as k_B for control buffers and k_L for liposomal suspensions.

Biphasic rates, observed in liposomes containing PC, were described by

$$F = f_A e^{-\alpha t} + f_B e^{-\beta t} \quad (5)$$

where F is the fraction remaining at time t; f_A and f_B are the ancitabine fractions lost in accordance with the rapid (α) and the slow (β) rate constants respectively. The values for f_A, f_B, α and β were obtained using nonlinear regression. The sum of the ancitabine and cytarabine concentrations throughout all reactions was equal to the initial concentration.

Influence of the phase transition temperature. The stabilization ratio, which reflects liposomal protection from the external environment, was defined as

$$\text{stabilization ratio} = k_B/k_L \text{ or } k_B/\beta \quad (6)$$

Table I lists hydrolysis rate constants and stabilization ratios for ancitabine encapsulated in various liposomes. The phase transition temperature for each of the phospholipids is (°C): ~4° (PC); ~23° (DMPC); ~41° (DPPC); ~41° (DPPG); ~55° (DSPC).[6,7] The alkaline suspension of liposomes containing ancitabine encapsulated in an acidic solution showed that DPPC and DSPC significantly increased stability as indicated by their stabilization ratios of 22. In contrast, stabilization was not exhibited by liposomes with PTT values below the ambient temperature of 25° such as PC and DMPC.

TABLE 1

Ancitabine Hydrolysis Rate Constants (min^{-1}) Following Dispersion of Liposomes Containing Acidic Solutions (0.15 M Acetate Buffer at pH 4.7) in Bicarbonate Buffer (0.1 M at pH 9.5) and Stabilization Ratios (k_B/k_L or k_B/β) at 25°C.

Liposome Component	$10^3 k_L$	k_B/k_L
DMPC	14.1	1.03
DPPC	0.647	22.4
DSPC	0.677	21.4
DMPC:CHOL	7.85	1.85
DPPC:CHOL	3.80	3.82
DPPC:DPPG:CHOL	5.10	2.84
DSPC:CHOL	2.40	6.04
	$10^3 \alpha$	$10^3 \beta$	k_B/β
PC	132	10.5	1.38
PC:CHOL	55.0	8.70	1.67

At temperatures below the PTT values, the interactions between hydrophobic acyl chains of adjacent phospholipids produce tightly ordered packing in the lipid bilayer.[7-10] At temperatures higher than the PTT values, these interactions are greatly reduced thus increasing bilayer mobility, decreasing bilayer thickness and increasing the hydration per phospholipid

molecule.[8-11] This enhanced movement of the matrix increases permeability. Deamer and Bramhall[11] have shown that the rate of collapse of a pH gradient across DPPC bilayers increases with temperature showing a sharp rate of increase at the PTT. They note that the membrane thickness of DPPC bilayers decreases by at least 50% upon passing above the PTT.

Although suspensions of DPPC liposomes containing Tris-buffered solutions (pH ◻ 8.62) showed biphasic kinetics, the contribution of the α phase to the total area under the curve was less than 5 percent. Using the β value for comparison, this change in the internal pH from 4.7 to 8.6 reduced the stabilization ratio from ~22 to ~3. This study simulates the behavior that would be expected if instant decay of the pH gradient to within one unit of the external pH occurred when the liposomes were challenged. Therefore, the original 22-fold increase must be due in part to successful maintenance of a pH gradient rather than a direct interaction with the liposomal structure.

Influence of cholesterol. Figure 2 compares the stabilization ratios in the presence of 33 mol% cholesterol to those in its absence. The ratios in the presence of cholesterol increase less dramatically with an increase in the PTT values of the phospholipids than do those in the absence of cholesterol. For example, the 22-fold increase in stabilization with DPPC and DSPC liposomes were reduced to a 4- to 6-fold increase in the presence of cholesterol.

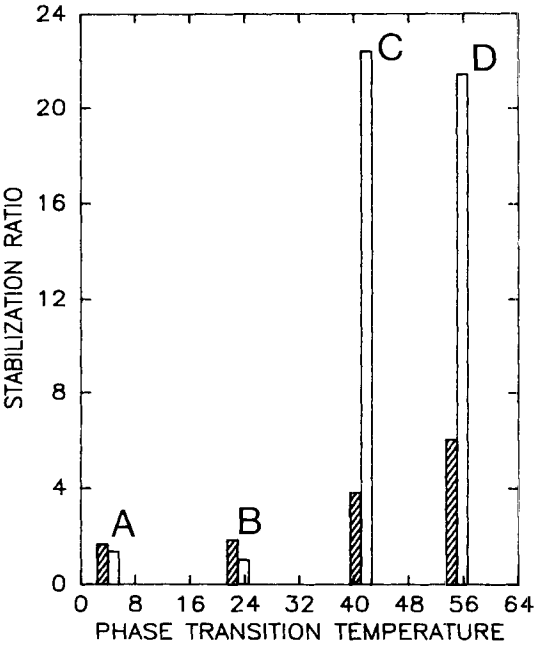

FIGURE 2

Stabilization ratios (equation 6) at 25° for ancitabine encapsulated in PC (A), DMPC (B), DPPC (C), and DSPC (D) liposomes with (shaded histogram) and without (open histogram) 33 mol% cholesterol as a function of the phase transition temperature of the phospholipid in each type of vesicle.

Stabilization ratios for ancitabine in DPPC liposomes containing varying amounts of cholesterol are shown in Fig. 3. Ancitabine encapsulation is constant at 32%. Addition of 16.6 mol% cholesterol results in a sharp decrease in the stabilization ratio from 22 to 4. Increasing cholesterol content does not produce further reduction.

Interfacial phospholipid-cholesterol interactions reduce the hydrophobic interactions between adjacent phospholipid molecules

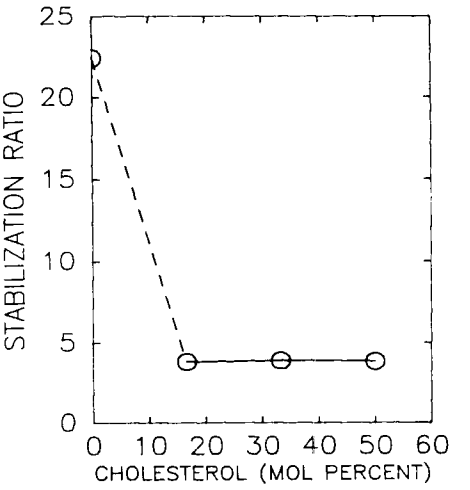

FIGURE 3

Stabilization ratios at 25° for ancitabine encapsulated in DPPC:CHOL liposomes as a function of the molar content (mol%) of cholesterol.

thus increasing the fluidity of the lipid bilayer.[12,13] Although the exact nature of this interaction is not understood, this disruptive effect could increase permeability. This is consistent with the greater release of ancitabine from the DPPC:CHOL liposomes at lower temperatures relative to vesicles containing DPPC alone (Fig. 4).

Effect of temperature. The rate constants for hydrolysis in DPPC liposomes converge with those in control buffers at approximately the PTT when plotted as a function of temperature (Fig. 5A). The stabilization ratios therefore decrease from ~22 at 25°C to unity at the PTT. In contrast, hydrolysis rate constants for ancitabine encapsulated in DPPC:DPPG:CHOL liposomes do not

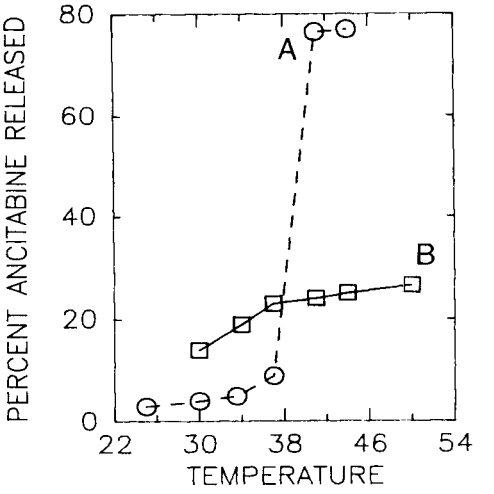

FIGURE 4

The percentage of ancitabine released during 5 minutes from (A) DPPC liposomes and (B) DPPC:CHOL liposomes as a function of temperature.

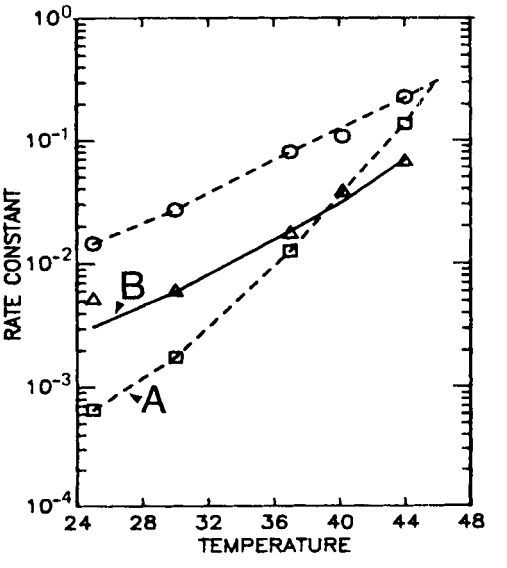

FIGURE 5

Semilogarithmic plot of rate constants (min^{-1}) for the hydrolysis of ancitabine as a function of temperature following acidic encapsulation in (A) DPPC liposomes (□) and (B) DPPC:DPPG:CHOL liposomes (Δ) with subsequent dispersion in buffer to provide pH 9.4. The control solution (○) is bicarbonate (0.1 M.):acetate (0.15 M.) at a 9:1 ratio with a pH = 9.4.

converge with those in the control buffers when plotted as a function of temperature (Fig. 5B) and the stabilization ratios remain constant at ~3.7. Hydrolysis studies were conducted at ~1-5°C above and below the PTT for three of the liposome preparations. The stabilization ratio for DPPC above its PTT is 1.6 whereas it is ~6 below the PTT. DMPC liposomes had a ratio of ~1 above and 2.3 below while DPPC:DPPG:CHOL had nearly equal ratios of 3.5 and 4.7.

The percentage of ancitabine released in 5 minutes as a function of temperature is presented in Fig. 4. The percentage released from DPPC liposomes shows a sharp increase at the PTT value (41°C). Similar results were obtained for 15 minute release studies. The addition of 33 mol% cholesterol resulted in greater release at lower temperatures with a moderate increase over the temperature range and a maximum of 25% release at 50°C.

Reports containing X-ray diffraction and NMR data have indicated that molecular motion of the acyl chains in the lipid bilayer gradually increases with increasing temperature until significant molecular movement occurs at the PTT.[7] At temperatures below the PTT, increasing mobility would increase permeability. This is consistent with the observed temperature-dependence for release and for stabilization.

At temperatures above the PTT, the absence of stabilization is probably due to increased membrane permeability. This is evidenced by the fact that during a 5 minute period, 70-80% of

the ancitabine was released from the DPPC liposomes. The hydroxyl ion, being smaller in size, would be expected to have even greater permeability. Previous reports concur that passage of various molecules through the bilayer is dramatically increased near the PTT.[14,15]

In contrast, liposomes containing 33 mol% cholesterol exhibit modest stabilization ratios (~3.7) which remain constant throughout the temperature range of the study (Fig. 5B). No significant differences were observed above and below the phospholipid PTT. Cholesterol reportedly has been described as having a 'modulatory' influence on the lipid bilayer.[8,12] Below the PTT of the pure phospholipid, cholesterol has a liquefying effect on the bilayer by reducing interactions between adjacent lipid molecules. The bilayer therefore remains in a so-called 'intermediate fluid' condition both below and above the PTT and the permeability is therefore less sensitive to temperature changes.

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