Diplasmenylcholine–Folate Liposomes: An Efficient Vehicle for Intracellular Drug Delivery†

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Diplasmenylcholine−Folate Liposomes: An Efficient Vehicle for Intracellular Drug Delivery†

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Abstract: Most pharmaceutical and gene therapy applications of targeted liposomes presently suffer from inefficient contents delivery to the cytoplasm of target cells. We report a plasma-stable liposome, composed of synthetic, naturally occurring diplasmenylcholine (1,2-di-*O*-(*Z*-1′-hexadecenyl)-*sn*-glycero-3-phosphocholine; DPPlsC), that rapidly and efficiently releases its contents at endosomal pHs. Acid-catalyzed hydrolysis of these liposomes produces glycerophosphocholine and fatty aldehydes, leading to greatly enhanced liposome permeability ($t_{50\%\ release} \cong 1-4$ h between pH 4.5−5.5) when >20% of the vinyl ether lipid has been hydrolyzed. Plasma stability of nonhydrolyzed 9:1 DPPlsC/dihydrocholesterol liposomes exceeds 48 h at 37 °C, pH 7.4 in 50% serum; pure DPPlsC liposomes remain stable in 10% serum under the same conditions. Fluorescence assays of KB cells treated with 99.5:0.5 DPPlsC/DSPE−PEG3350−folate liposomes containing encapsulated propidium iodide (PI) indicate that 83% of the PI escapes the endosomal compartment within 8 h to produce intensely stained nucleii. The IC_{50} value of 1-β-arabinofuranosylcytosine (Ara-C) encapsulated in DPPlsC/DSPE−PEG3350−folate liposomes is 0.49 μM in KB cell cultures, a ∼6000-fold enhancement in cytotoxicity compared with free drug (2.8 mM). Empty DPPlsC/DSPE−PEG3350−folate liposomes had no effect on DNA synthesis, indicating that DPPlsC and its degradation products are benign to cell function at these lipid concentrations. Our results suggest that concurrent application of selective targeting and membrane translocation mechanisms in drug carriers can significantly increase their efficacy.

Introduction

Liposome-encapsulated drugs, presently used for the clinical treatment of cancers and fungal infections, are undergoing accelerated development for applications in gene therapy.[1,2] These applications utilize actively-[3−11] and passively-[12−17] targeted liposomes as the drug carrier because of their site-

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† Abbreviations: AraC, 1-β-arabinofuranosylcytosine; DHC, 5a-cholestane-3b-ol (dihydrocholesterol); DPPlsC, 1,2-di-*O*-1′-*Z*-hexadecenyl-*sn*-glycero-3-phosphocholine; EPC, egg phosphatidylcholine; FDMEM, folate-deficient modified Eagle's medium; HIFCS, heat-inactivated fetal calf serum; IC_{50}, 50% growth inhibition concentration; PBS, phosphate buffered saline (150 mM NaCl, 2.0 mM Na_2HPO_4, pH 7.4); PI, propidium iodide.

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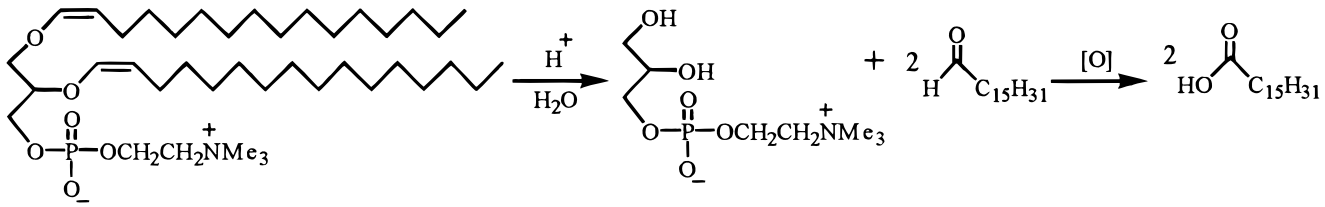

Figure 1. Acid-catalyzed hydrolysis of 1,2-di-*O*-(*Z*-1′-hexadecenyl)-*sn*-glycero-3-phosphocholine (DPPlsC) to give the degradation products glycerophosphocholine, hexadecanal, and hexadecanoic acid.

selectivity, biocompatibility, high drug/lipid ratios, and blood clearance characteristics. Unfortunately, existing methods for transporting the liposomal contents to the target cell cytoplasm are either inefficient or lack sufficient plasma stability for in vivo applications. This obstacle is especially problematic for the cytoplasmic delivery of peptides, antisense oligonucleotides, and gene constructs since (i) their size and hydrophilicity prevents efficient cytoplasmic delivery to the desired target unless specific membrane fusion and/or cytoplasmic release mechanisms[18,19] have been incorporated, (ii) the liposomal cargo not rapidly discharged into the cytoplasm is typically delivered to lysosomes where it is degraded, and (iii) the cationic lipids and polymers that are used for nucleic acid transfer are often cytotoxic and/or have limited plasma stability. Notable exceptions are the first-pass use of adenovirus vectors[20] or fusogenic "virosomes" derived from influenza virus envelopes;[21] however, questions regarding the safety and processability of these carriers remain unanswered.

We report a hybrid liposome system, incorporating both folate receptor-mediated cell targeting (via a DSPE−PEG3350−folate conjugate[22,23]) and a pH-sensitive trigger using the naturally occurring vinyl ether-linked phospholipid diplasmenylcholine (1,2-di-*O*-(1′-*Z*-hexadecenyl)-*sn*-glycero-3-phosphocholine; DPPlsC[24,25]), that was designed to obviate these problems and efficiently deliver its contents to the cytoplasm of KB cells, a human nasopharyngeal epidermal carcinoma cell line, upon exposure to the acidic endosomal compartment. Acid-catalyzed hydrolytic triggering in this system is analogous to the photoinduced triggering previously reported by this laboratory for plasmenylcholine[18,26] and diplasmenylcholine[27,28] liposomes; both systems utilize chemical activation mechanisms to produce lipid phase transitions by degrading lamellar phase-forming phospholipids to a pair of single-chain surfactant products (Figure 1).

Results and Discussion

Diplasmenylcholine Liposome Hydrolysis and Its Influence on Calcein Release. Acid-catalyzed release of calcein from DPPlsC liposomes suspended in buffer was studied as a model system for endosomal release. No detectable calcein release occurs from DPPlsC liposomes maintained at pH 7.4, 37 °C for 48 h, in contrast to their leakage properties at pH 4.5 where the half-time for release ($t_{50\%\ \mathrm{release}}$) is 76 min. Calcein leakage rates increase with decreasing pH (Figure 2a and Table 1) and with the extent of DPPlsC hydrolysis at pH 4.5 (Figure 2b,c), however, they decrease with increasing mole fraction of the saturated cholesterol derivative, 5a-cholestane-3b-ol (dihydrocholesterol, DHC[29]) (Figure 2d). Hydrolysis rates of DPPlsC, monitored by HPLC-ELS analysis, were independent of DHC content, suggesting that a critical extent of diplasmenylcholine degradation is required before the onset of rapid calcein leakage occurs (20 to ∼80% hydrolysis, depending on DHC content; Figure 2c). DPPlsC hydrolysis kinetics at pH 4.5, a pH regime that can occur within the endosomes of KB cells,[30] are pseudo-first-order ($k_{obs} = 6.3 \times 10^{-5}\ s^{-1}$ at pH 4.5). Calcein release rates, however, are nonlinear, with dramatic increases in leakage rate occurring between 15 and 30% of lipid hydrolysis (Figure 2b,c). These results suggest that membrane destabilization occurs only after a critical concentration of diplasmenylcholine degradation products have accumulated within the bilayer. The critical behavior of this coupled hydrolysis-leakage process and the independence of the DPPlsC hydrolysis rate on the mol % DHC in the bilayer are features shared by semisynthetic plasmenylcholine liposomes undergoing acid-catalyzed hydrolysis.[31] ^{1}H NMR and mass spectra of the DPPlsC hydrolysis products, isolated by Bligh−Dyer extraction of the acidic liposome solutions, indicate that the predominant lipophilic degradation products are hexadecanal and hexadecanoic acid. These results indicate that palmitic acid and glycerophosphocholine are the terminal acid-catalyzed hydrolysis products of DPPlsC as illustrated in Figure 1. Partially hydrolyzed lysolipid intermediates (e.g., 1-hydroxy-2-(1′-*Z*-hexadecenyl)-*sn*-glycero-3-phosphocholine, 1-(1′-*Z*-hexadecenyl)-2-hydroxy-*sn*-glycero-3-phosphocholine, or the cyclized isomer, 1,2-hexadecylacetal-*sn*-glycero-3-phosphocholine), in principle, may also participate in bilayer destabilization during the course of the hydrolysis reaction; however, since these

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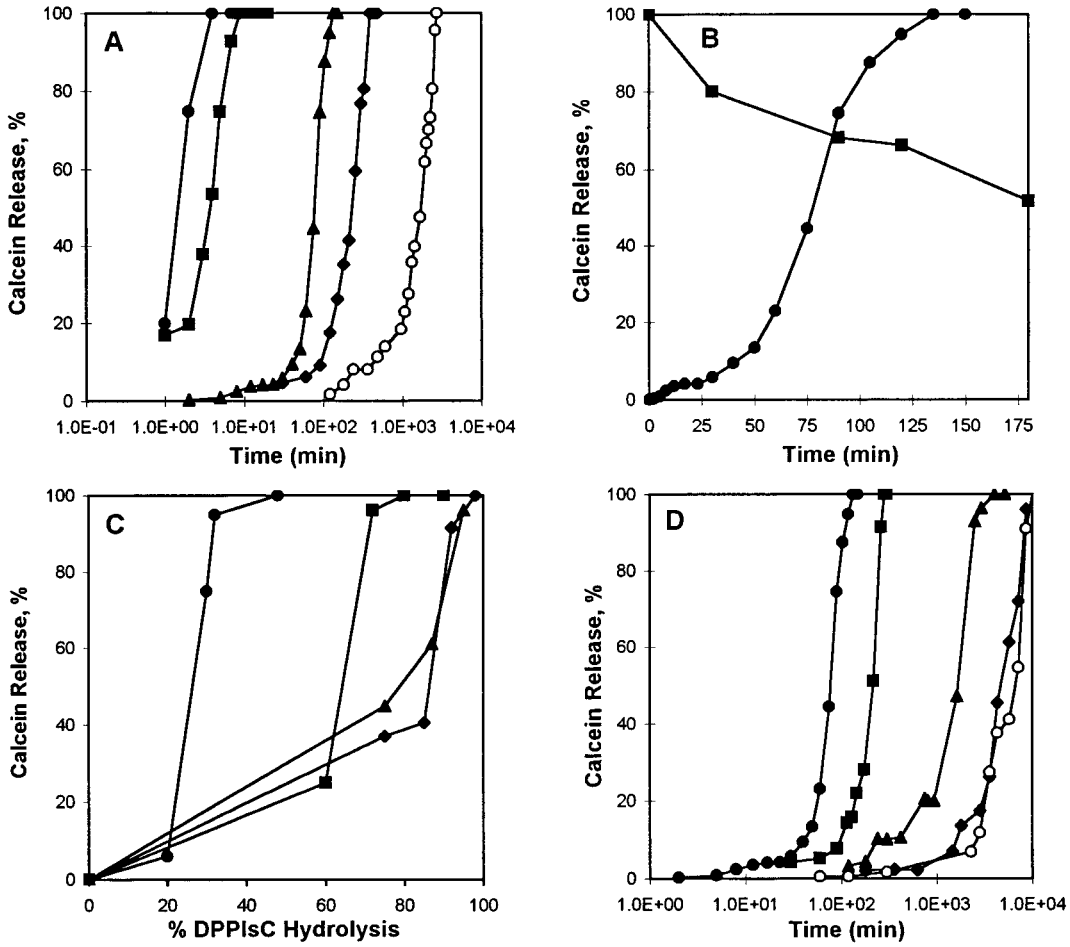

Figure 2. (a) Calcein release rates at 37 °C from DPPlsC liposomes in different buffers.[26,31] ●, pH 2.3; ■, pH 3.2; ▲, pH 4.5; ◆, pH 5.3; ○, pH 6.3. (b) Calcein release rate (●) and DPPlsC hydrolysis[31] rate (■) from DPPlsC liposomes at pH 4.5, 37 °C. (c) Correlation between % calcein released and the extent of DPPlsC hydrolysis in liposomal dispersions at pH 4.5, 37 °C. ●, pure DPPlsC; ■, 8:2 DPPlsC/DHC; ▲, 7:3 DPPlsC/DHC; ◆, 6:4 DPPlsC/DHC. Note that as the DHC content increases, more extensive DPPlsC hydrolysis is required to effect 50% calcein release. (d) Calcein release rates at 37 °C from DPPlsC/DHC liposomes in citrate buffer (pH 4.54) containing different molar ratios of DPPlsC and DHC. ●, pure DPPlsC; ■, 9:1 DPPlsC/DHC; ▲, 8:2 DPPlsC/DHC; ◆, 7:3 DPPlsC/DHC; ○, 6:4 DPPlsC/DHC.

Table 1. pH Dependence of 50% Release Time

pH	$t_{50\%\ \text{release}}$ (min)
2.3	1.5
3.2	3.6
4.5	76
5.3	230
6.3	1740

species were not detected by HPLC analysis,[32] we believe that they are less important in promoting membrane permeability than the terminal hydrolytic product, hexadecanoic acid.

Plasma Stability of Diplasmenylcholine Liposomes. Unlike many pH-sensitive liposome formulations,[18,19] the plasma stability of DPPlsC liposomes at 37 °C is quite good. Pure DPPlsC liposomes do not leak calcein upon exposure to 10% heat-inactivated fetal calf serum (HIFCS) for up to 48 h. The addition of ≥10% DHC to the DPPlsC membrane further stabilizes these liposomes in 50% HIFCS over the same time period (Table 2). Incorporation of 0.5 mol % DSPE−PEG3350−folate within the DPPlsC membrane also did not affect the plasma stability of the liposomes. These results suggest that DPPlsC liposomes are sufficiently plasma-stable for in vitro drug delivery applications.

Table 2. Liposome Stability at pH 7.4, 37 °C

liposome type	50% HIFCS 24 h	50% HIFCS 48 h	10% HIFCS 48 h
DPPlsC	27%	33%	0
9:1 DPPlsC/DHC	0	0	0
8:2 DPPlsC/DHC	0	0	0
7:3 DPPlsC/DHC	0	0	0
6:4 DPPlsC/DHC	0	0	0

[a] % release values are ±5%.

Cytoplasmic Delivery of Propidium Iodide. The ability of folate-targeted DPPlsC/DHC liposomes to promote endosomal delivery of hydrophilic compounds to the cytoplasm of KB cells was evaluated by fluorometric assay using propidium iodide (PI) as a fluorescent probe.[30,33] PI fluorescence (λ_{ex} = 540 nm, λ_{em} = 615 nm) increases approximately 50-fold upon binding to RNA or DNA. This property makes it useful for endosomal release assays, since cellular fluorescence at 615 nm from internalized PI effectively arises only after it has escaped the endosome and binds to nucleic acids. PI release kinetics revealed that 83% of the encapsulated PI escaped both the liposomal and endosomal compartments within 8 h when ≤10

(32) Lysolipids are readily detected by our HPLC-ELS methodology.[31] Since they were not observed by HPLC-ELS during the course of the hydrolytic reaction and were also not detected by ^{1}H NMR in the isolated organic soluble fraction from Bligh−Dyer extraction, these partially hydrolyzed species must be cleaved more rapidly than their diplasmenylcholine precursors.

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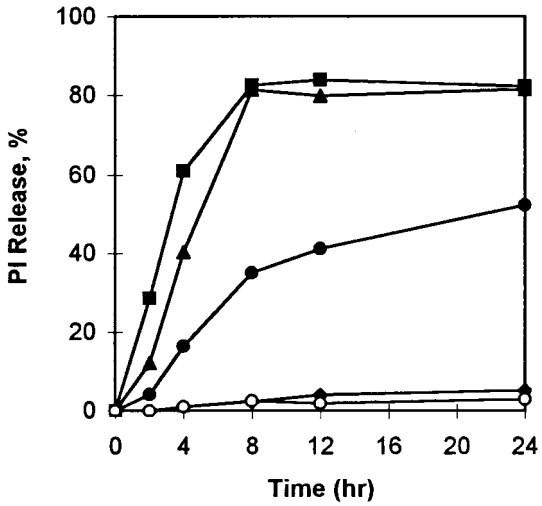

Figure 3. Propidium iodide release kinetics in KB cells using liposomes targeted with 0.5% DSPE−PEG3350−folate lipid.[22,23] ◆, egg phosphatidylcholine (EPC) liposomes (pH-insensitive control); ■, pure DPPlsC liposomes; ▲, 9:1 DPPlsC/DHC liposomes; ●, 8:2 DPPlsC/DHC liposomes; ○, 9:1 DPPlsC/DHC liposomes in the presence of 25 μM monensin.

mol % DHC was present in the DPPlsC membrane; 36% release occurred within 8 h (53% after 24 h) when the DHC content was increased to 20 mol % (Figure 3). Endosomal unloading of PI was also confirmed by fluorescence microscopy (Figure 4), where intense staining of nucleii and nucleoli indicates that the released PI has also penetrated into the nucleus. Both the extent and rate of PI release were greater for DPPlsC liposomes than for folate-targeted EPC vesicles containing the pH-sensitive peptide EALA either covalently attached (9% release in 8 h; 20% in 24 h) or added to the external medium (4% release in 8 h; 13% in 24 h).[33] Control experiments, using KB cells treated with DPPlsC/DSPE−PEG3350−folate/PI_{in} liposomes in the presence of the endosomal acidification inhibitors monensin (25mM) and chloroquine (50mM), indicated that <5% PI escaped into the cytoplasm, when monitored for up to 24 h after liposomal treatment (Figure 3). These results strongly suggest that an acidic endosomal compartment is necessary to trigger cytoplasmic contents delivery from DPPlsC liposomes, presumably via the involvement of fatty aldehydes and acids derived from diplasmenylcholine hydrolysis.

Cytoplasmic Delivery of 1-β-Arabinofuranosylcytosine (Ara-C). The ability of folate-targeted 9:1 DPPlsC/DHC liposomes to enhance cytoplasmic delivery of the cytotoxic antimetabolite drug, Ara-C,[34] was monitored by [^{3}H]thymidine incorporation assay (Figure 5). These liposomes exhibit a ~6000-fold enhancement of DNA synthesis inhibition relative to free Ara-C in KB cell cultures (IC_{50} = 490 nM and 2.8 mM for DPPlsC/folate liposomal and free Ara-C, respectively). DPPlsC/DHC/DSPE−PEG3350−folate (9:1:0.05) liposomes containing Ara-C represent an improvement over transferrin-conjugated, Ara-C containing pH-sensitive phosphatidylethanolamine liposomes[35] by a factor of 70, pH-sensitive immunoliposomes[36,37] by a factor exceeding 1000, and liposomal Ara-C prodrugs by a factor of approximately 400.[38] Inhibition of DNA synthesis was not observed in KB cells treated with empty (control) DPPlsC/DSPE−PEG3350−folate liposomes, indicating that neither the lipid nor its degradation products have a significant effect on this cellular function at the lipid concentrations used.

Conclusion

Allen and co-workers have demonstrated the efficacy of passively targeted, Ara-C-containing sterically stabilized liposomes in a murine model and attributed their increased therapeutic effect, relative to free Ara-C, to a sustained extracellular release mechanism from these formulations.[39] Our results demonstrate that endosomal triggering of DPPlsC liposomes provides a fast, efficient, and practical method for intracellular delivery of Ara-C and other water-soluble materials,[40] which significantly improves their biological activity. Synergistic action of receptor-mediated cell targeting and acid-catalyzed endosomal escape may offer greatly enhanced drug efficacy for many hydrophilic, nonmembrane permeable compounds. Experiments designed to both elucidate the endosomal escape mechanism and explore the applications of spontaneous liposomes[41] composed of DPPlsC:PEG formulations for drug delivery and gene transfer are in progress.

Experimental Methods

Liposome Preparation. Calcein-containing Liposomes without DHC. Pure DPPlsC liposomes were prepared by hydrating 3.4 mg of DPPlsC powder in 1 mL of calcein solution (prepared by dissolving 93 mg of calcein in 3.0 mL of 0.3 M NaOH to give a final concentration of 50 mM, pH 12.5) using five LN_2 freeze−thaw-vortex cycles. The lipid suspension was then extruded at 50 °C through two 100 nm track-etch polycarbonate membrane filters.[42] Extraliposomal calcein was removed by using a single pass through a 40-cm Sephadex G-50 gel column equilibrated with 150 mM NaCl. The fraction eluting at the void volume was collected, stored at 8 °C, and used within 24 h. This general procedure was used for all liposome preparations, except as noted below.

Calcein-containing DPPlsC/DHC Liposomes. DPPlsC and DHC, dissolved in chloroform that had been pre-filtered through a 1'' plug of anhydrous sodium carbonate to remove traces of acid and water, were evaporated with a stream of dry N_2 gas, followed by evacuation at <200 μm for at least 4 h to give thin lipid films. Liposomes were prepared as described above by hydrating these films in the presence of a 50 mM calcein solution, followed by extrusion and removal of the extravesicular calcein by gel filtration.

DPPlsC Liposomes Containing PI or Ara-C. DPPlsC (0.020 mmol), DSPE−PEG3350−folate (0.10 mmol), and DHC (0, 0.002, and 0.004 mmol) in prefiltered $CHCl_3$ (see above) were mixed and evaporated to a thin film. The dry lipid film was hydrated in the presence of 10 mg/mL PI or Ara-C in phosphate-buffered saline (150 mM NaCl, 2.0 mM Na_2HPO_4, pH 7.4, PBS) and extruded as described above. Unencapsulated solutes were removed using a single pass through a 40-cm Sephadex G-50 column equilibrated and eluted with PBS.

Calcein Release Assay. Calcein-containing liposomes (1.0 mL) were mixed at 37 °C with 1.0 mL of 20 mM phosphate (pH 6.3 and 5.3), citrate (pH 4.5), or oxalate (pH 3.2) buffer in 150 mM NaCl; HCl in 150 mM NaCl was used for pH 2.3 hydrolyses. Calcein

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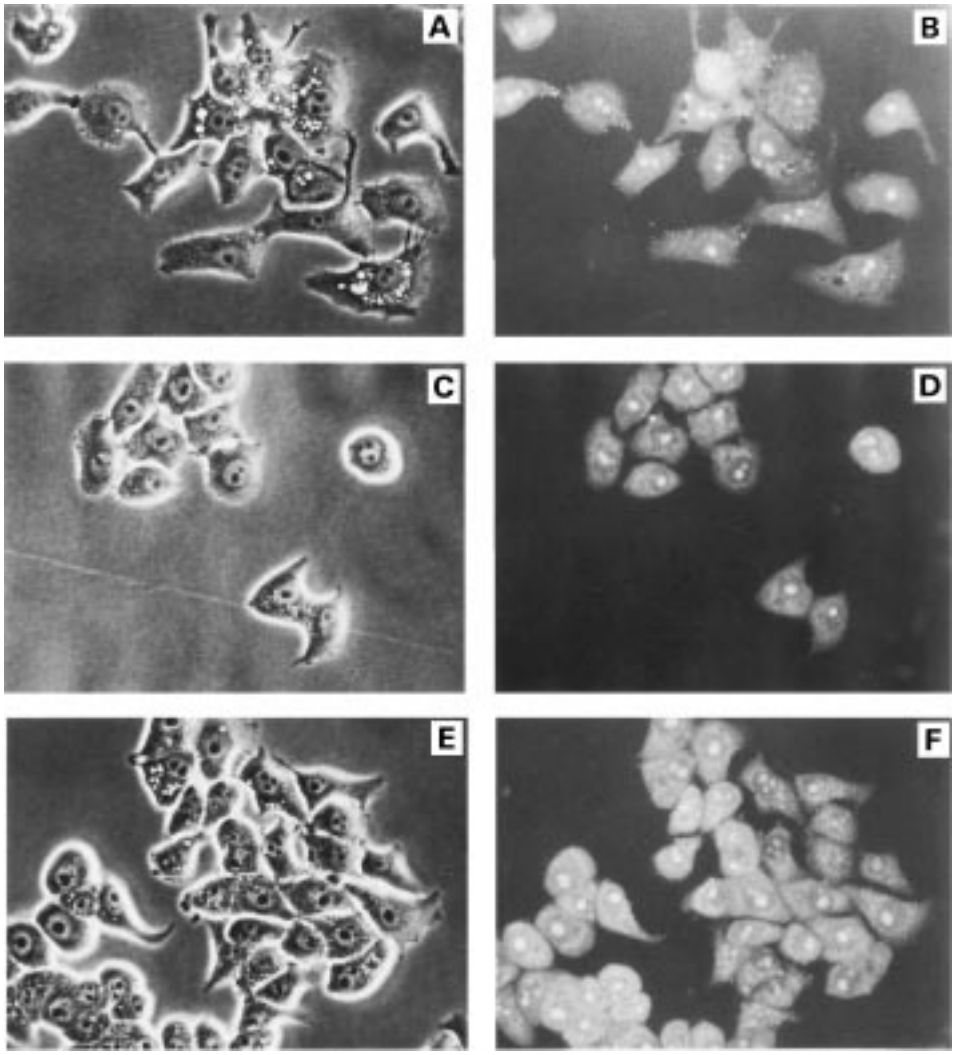

Figure 4. Micrographs of KB cells treated for 2 h with DPPlsC/folate/PI liposomes as in Figure 3, washed with phosphate-buffered saline, pH 7.4 (PBS) to remove folate-targeted liposomes, and visualized at various times. Phase contrast micrographs taken 4 h (A), 8 h (C), and 24 h (E) after PBS wash. Fluorescence micrographs taken at 4 h (B), 8 h (D), and 24 h (F) after addition of DPPlsC/DSPE−PEG3350−folate/PI_{in} liposomes.

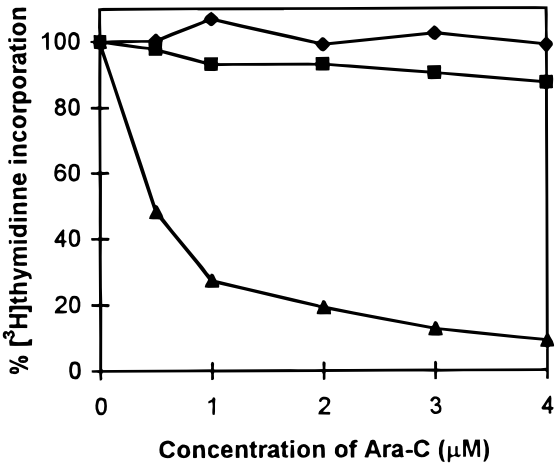

Figure 5. Cytotoxicity of Ara-C in KB cell cultures. Free Ara-C (◆); Ara-C encapsulated in EPC/DSPE−PEG3350−folate liposomes (■); 9:1 DPPlsC/DHC/DSPE−PEG3350−folate liposomes (▲).

fluorescence dequenching was monitored as a function of time by diluting 50 μL aliquots of the hydrolysis mixture into 2 mL of 150 mM NaCl/20 mM HEPES, pH 7.4 prior to measurement of the calcein fluorescence intensity (λ_{ex} = 490 nm; λ_{em} = 518 nm) using a Perkin-Elmer MPF-66 spectrofluorimeter. The extent of liposomal leakage was then compared with the 100% release value (determined by adding Triton X-100 to the diluted liposome sample) using the ratio method.[26]

Analysis of DPPlsC Liposome Hydrolysates. At various time points, an 80-μL aliquot of the liposome hydrolysis solution was withdrawn and added to a mixture of 200 μL of methanol and 100 μL of chloroform. After the solution was mixed thoroughly by vortexing, an additional 80 μL of water and 100 μL of chloroform were added. The sample was again vortexed and then centrifuged for 3 min at 10 000 rpm. A 25-μL sample of the chloroform phase was analyzed with a Waters model 510 HPLC equipped with a Sedex 55 evaporative light scattering detector. DPPlsC hydrolysis rates were determined using a normal phase column (3.9 × 300 mm μ-Porasil silica column) and 4:5:1 (v/v) hexanes/methanol/water as eluent (2.2 mL/min flow rate)[31] by monitoring the disappearance of DPPlsC as a function of hydrolysis time. No peaks due to the accumulation of lysoplasmenylcholine intermediates (i.e., either the *sn*-1 or *sn*-2 monohydrolysis products) or the 2-pentadecyl-1,3-dioxolane sideproduct (i.e., the isomeric, internally trapped cyclic acetal monohydrolysis product) were observed in any of the hydrolysis samples analyzed by this technique.

Fully hydrolyzed DPPlsC liposome samples (as determined by the disappearance of the DPPlsC peak in HPLC) were extracted with chloroform (3×), evaporated, and redissolved in sodium carbonate-filtered $CDCl_3$ for ^{1}H NMR analysis. NMR analysis of the liposome samples immediately at the end of the hydrolysis reaction revealed that the $CHCl_3$/MeOH extracted material contained no phosphocholine resonances and two new resonances at 2.3 ppm (2H) and 8.4 ppm (1H). If the samples were lyophilized >10 h after completion of the hydrolysis

reaction, the 8.4 ppm resonance was replaced by a broad signal centered at 10.5 ppm (1H). These products were identified by FAB-MS (DTT matrix) as hexadecanal (*m*/*z* 241, M + 1) and hexadecanoic acid (*m*/*z* 257, M + 1), respectively. HPLC analysis of authentic hexadecanoic acid samples revealed no detectable peaks under the conditions described above, presumably due to elution at the column void volume.

Evaluation of DPPlsC Liposome Plasma Stability. Calcein-containing DPPlsC liposomes dispersed in 20 mM HEPES/150 mM NaCl, pH 7.4 were mixed with HIFCS in volume/volume ratios of 9:1 (10% serum) and 1:1 (50% serum) at 37 °C. Aliquots (0.1 mL) were withdrawn as a function of time, diluted in 2.0 mL of HEPES buffer, and the extent of calcein leakage was measured as described above.

Propidium Iodide Delivery to KB Cells. KB cells were cultured for at least 5 weeks in folate-deficient modified Eagle's medium (FDMEM) to establish folate-deficiency before beginning the experiments. These cells were then incubated (33 mm culture dishes, ~2.5 × 10^6 cells/dish, ~85% confluence) in 1.0 mL of FDMEM with 0.20 mL of the DPPlsC/DSPE−PEG3350−folate/5 mM PI_{in} liposomes for 2 h at 37 °C. After the cells were washed thoroughly with PBS to remove any unbound liposomes, 1.0 mL of fresh FDMEM was added, and the cells were re-incubated for up to 24 h. The cells were washed with PBS 0, 2, 4, 8, 12, and 24 h after treatment with the PI-containing liposomes, suspended in nonenzymatic cell dissociation solution, and the PI fluorescence measured (λ_{ex} = 540 nm, λ_{em} = 615 nm). PI escape from the endosomes was quantitated using the expression, % release = $[(F_t - F_0)/(F_{max} - F_0)] \times 100$, where F_t = PI fluorescence emission intensity at time *t*, F_0 = background fluorescence emission intensity at time = 0 h of cells not treated with PI-containing liposomes, F_{max} = PI fluorescence emission intensity observed after sonicating the cells for 15 min, and F'_0 = background fluorescence of untreated cells disrupted with a 15-min sonication. (Control experiments showed that longer sonication periods, freeze−thawing + 15 min sonication, or 0.1 M Triton ×100 treatment gave essentially the same extent of cellular PI fluorescence as the method described above.) Phase contrast and fluorescence micrographs were taken at 4, 8, and 24 h to visualize the extent of PI release from the endosomes. Endosomal acidification inhibition control experiments were performed in the same manner, except that final medium concentrations of 25 μM monensin (obtained by adding 20 μL of 1.25 mM monensin stock in PBS) or 50 μM chloroquine (obtained by adding 20 μL of 2.5 mM chloroquine stock in PBS) were maintained during the 2 h liposome-containing and 24 h liposome-free incubations.

Ara-C Cytotoxicity in KB Cell Cultures. KB cells were plated to 50% confluence in 24-well culture plates before treatment with free Ara-C, Ara-C encapsulated in 6:4 EPC/cholesterol/DSPE−PEG3350−folate liposomes, or 9:1 DPPlsC/DHC/DSPE−PEG3350−folate liposomes. Liposomes were prepared as described above, except that the lipids were hydrated in an Ara-C solution (PBS, pH 7.4); drug concentration after gel filtration = 500 μM, yielding a [drug]/[lipid] ratio of 1:65. After 4 h exposure to the Ara-C suspensions at various concentrations, the cells were washed extensively with PBS and incubated in fresh FDMEM in the presence of [^{3}H]thymidine (2 μCi/well). After 24 h, the DNA was precipitated with trichloroacetic acid and dissolved in 2 M NaOH, and [^{3}H]thymidine incorporation was measured by scintillation counting.

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