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Research Paper

Transition Metal-Mediated Liposomal Encapsulation of Irinotecan (CPT-11) Stabilizes the Drug in the Therapeutically Active Lactone Conformation

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Purpose. To determine whether entrapped transition metals could mediate the active encapsulation of the anticancer drug irinotecan into preformed liposomes. Further, to establish that metal complexation could stabilize liposomal irinotecan in the therapeutically active lactone conformation.
Materials and Methods. Irinotecan was added to preformed 1,2-distearoyl-*sn*-glycero-phosphocholine/cholesterol (DSPC/chol) liposomes prepared in $CuSO_4$, $ZnSO_4$, $MnSO_4$, or $CoSO_4$ solutions, and drug encapsulation was determined over time. The roles of the transmembrane pH gradient and internal pH were evaluated. TLC and HPLC were used to monitor drug stability and liposome morphology was assessed by cryo-TEM.
Results. Irinotecan was rapidly and efficiently loaded into preformed liposomes prepared in unbuffered (~pH 3.5) 300 mM $CuSO_4$ or $ZnSO_4$. For Cu-containing liposomes, results suggested that irinotecan loading occurred when the interior pH and the exterior pH were matched; however, addition of nigericin to collapse any residual transmembrane pH gradient inhibited irinotecan loading. Greater than 90% of the encapsulated drug was in its active lactone form and cryo-TEM analysis indicated dark intravesicular electron-dense spots.
Conclusion. Irinotecan is stably entrapped in the active lactone conformation within preformed copper-containing liposomes as a result of metal–drug complexation.

KEY WORDS: copper; CPT-11; irinotecan; liposomes; metal complexation.

INTRODUCTION

Irinotecan (CPT-11) is a water-soluble analogue of camptothecin (CPT). Camptothecins exert their cytotoxic effects by stabilizing the covalent intermediate formed between topoisomerase I and DNA. This action prevents the re-ligation of the DNA strand, causing accumulation of DNA-topoisomerase I complexes which ultimately leads to apoptosis (1–3). The clinical introduction of irinotecan has had a significant impact on the treatment of cancer, particularly for the treatment of colorectal adenocarcinoma (4,5) and small cell lung cancer (SCLC; 6).

Irinotecan is subject to an enzymatic conversion that yields a number of metabolites (7), including SN-38 (8), which is reported to be up to 1,000-fold more active than irinotecan *in vitro* (9). Further, the anti-cancer activity of irinotecan (and SN-38) is dependent on the maintenance of the lactone ring in the closed form [Fig. 1; reviewed in (10)]. At physiological pH, equilibrium favors the inactive carboxy or ring-opened form and consequently, after intravenous administration, the lactone ring of irinotecan undergoes rapid hydrolysis (11). Resultantly, we are considerably interested in strategies designed to maintain irinotecan in its biologically active lactone form.

Liposomal formulation of camptothecins have been described previously and the distinct lipid and aqueous environments that characterize liposomes offer the potential to formulate a number of camptothecin derivatives from the poorly water soluble (12–14) to the freely soluble (15–17). In general, protection of the lactone ring in these formulations was attributed to partitioning of the drug into the lipid bilayer and/or isolation of the drug within the liposome's interior aqueous compartment that was maintained at low pH. For example, our laboratory as well as others have encapsulated topotecan (15,17,18) and irinotecan (16) in the acidic aqueous core of liposomes, thereby providing an internal milieu conducive to maintenance of the lactone form of the drug. These preparations have relied on pH gradients, generated by either an ammonium sulfate gradient (19), or the coupling of a manganese ion gradient and the divalent

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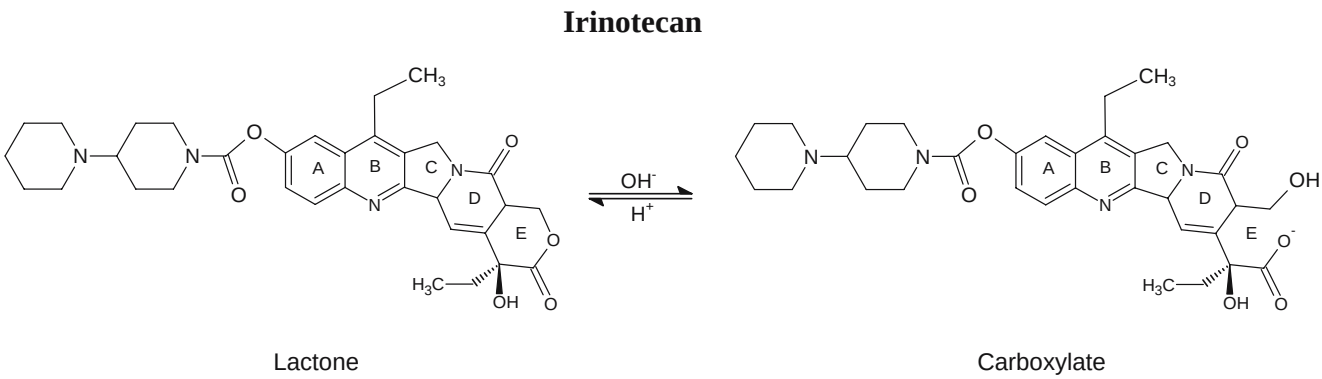

Fig. 1. The active lactone form of irinotecan is subject to a pH-dependent hydrolysis that yields the ring open inactive carboxylate.

cation ionophore A23187 (20), to drive the uptake of the drugs into preformed liposomes.

We have previously described an alternative mechanism for active drug loading of liposomes. A transmembrane Mn^{2+} ion gradient in the absence of any associated pH gradient was able to effectively mediate doxorubicin encapsulation via the formation of an intravesicular Mn^{2+}-doxorubicin 6-member chelate (21). The ability of transition metals to form coordination complexes with organic species, including drugs, is well established (22–25). Kuwahara *et al.* (26) described the efficient cleavage of DNA by a copper (II)-camptothecin (CPT) complex in combination with UV irradiation. The 1:1 CPT-Cu (II) complex was believed to be the result of copper ion coordination with the oxygen atoms of the CPT lactone ring. We hypothesized that a similar complexation reaction of CPT-11 with metals can be used to drive the liposomal encapsulation of the drug and stabilize the intravesicular irinotecan in the active lactone conformation.

This report describes the use of transition metals to mediate encapsulation of irinotecan into liposomes via complexation. Preformed liposomes containing copper (Cu), zinc (Zn), cobalt (Co) or manganese (Mn) were prepared and their uptake of irinotecan at different conditions was explored. The results indicate that irinotecan can accumulate in liposomes prepared in unbuffered $ZnSO_4$ and $CuSO_4$ solutions. Focusing on copper-mediated loading, drug accumulation occurred in the presence or absence of an initial transmembrane pH gradient. Cryo-TEM showed intravesicular electron-dense spots following encapsulation by copper-containing liposomes. Thin layer chromatography (TLC) and HPLC analysis of the drug-loaded liposomes indicated that the lactone ring of irinotecan was stabilized.

MATERIALS AND METHODS

Materials

Irinotecan hydrochloride (Camptosar®, Pharmacia) was purchased from the BC Cancer Agency Pharmacy. 1,2-distearoyl-*sn*-glycero-phosphocholine (DSPC) and cholesterol (chol) were obtained from Avanti Polar Lipids (Alabaster, AL). ^{3}H-cholesteryl hexadecyl ether (^{3}H-CHE) was purchased from PerkinElmer Life Sciences (Boston, MA). 8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt (HPTS; pyranine) was sourced from Invitrogen (Carlsbad, CA). All other chemicals used were analytical or HPLC grade.

Liposome Preparation

DSPC/chol (55:45 mol%) large unilamellar vesicles (LUVs) were prepared as previously described (27). Briefly, lipids were dissolved in chloroform at the required molar ratio, labeled with the non-exchangeable, non-metabolizable lipid marker ^{3}H-CHE, and dried to a thin film under a stream of nitrogen gas. Subsequently, the lipid was placed in a high vacuum for ≥3 h to remove any residual solvent. The lipid films were hydrated at 65°C by mixing with the appropriate solution prior to five cycles of freeze-and-thaw (5 min each, freezing in liquid nitrogen and thawing at 65°C). The multilamellar vesicle (MLV) suspensions were then extruded ten times through stacked polycarbonate filters of 0.08 and 0.1 μm pore size at 65°C (Extruder™, Northern lipids, Vancouver, BC, Canada). The resultant LUVs typically possessed mean vesicular diameters in the range 110 ± 30 nm as determined using quasi elastic light scattering (QELS) methods (NICOMP model 270 submicron particle sizer, Pacific Scientific, Santa Barbara, CA, USA). The LUVs external buffer was exchanged, using Sephadex G-50 size exclusion chromatography, with SHE buffer pH 7.5 (300 mM sucrose, 20 mM HEPES, 15 mM EDTA) or MES/HEPES buffer adjusted to pH 6.0, pH 6.5, pH 7.0, pH 7.5 (10 mM MES, 10 mM HEPES, 150 mM NaCl), or HBS buffer pH 7.5 (20 mM HEPES, 150 mM NaCl).

Accumulation of Irinotecan into DSPC/Chol Liposomes

Drug was incubated with liposomes at 50 or 60°C at a drug-to-lipid ratio of 0.2:1 (mol/mol) unless otherwise indicated. Uptake of the drug was determined at various time points by sampling 100 μl aliquots and separating encapsulated drug using 1 ml Sephadex G-50 spin columns equilibrated with the appropriate buffer (28,29). The excluded fractions, containing the liposomes, were analyzed in order to determine drug-to-lipid ratios. Lipid concentrations were measured using liquid scintillation counting (Packard 1900TR liquid scintillation analyzer). Irinotecan concentration was determined by measuring absorbance at 370 nm (Hewlett Packard UV–Vis spectrophotometer, model 8453).

Determination of Internal Liposome pH Following Irinotecan Encapsulation

DSPC/chol (55:45 mol%) liposomes were prepared as previously described. For this study the liposomes were formulated with the following internal buffers, both in the presence or absence of the fluorescent dye, HPTS (12.5 mM): 300 mM unbuffered $CuSO_4$; 300 mM $CuSO_4$/20 mM HEPES/TEA pH 6.0, pH 6.5, pH 7.0, pH 7.5; 300 mM citrate buffer pH 3.5; 20 mM HEPES buffer pH 7.5. Following extrusion the external buffer was exchanged with SHE pH 7.5 using column chromatography. Irinotecan was actively loaded into DSPC/chol liposomes prepared with 300 mM unbuffered $CuSO_4$ (initial unbuffered pH = 3.5) ± HPTS and 300 mM $CuSO_4$/20 mM HEPES/TEA pH 7.5 ± HPTS. Loading conditions were as previously described and the presence of HPTS did not impair the efficiency of irinotecan loading.

Fluorescent analysis of liposomes±HPTS was performed using a LS-50B luminescence spectrometer (PerkinElmer). The external buffer of the liposome solutions was exchanged to HBS pH 7.5 (20 mM HEPES, 150 mM NaCl) and diluted to a final lipid concentration of 0.5 mM in order to eliminate lipid-induced interference. Each preparation was subjected to an excitation scan (λ_{excit} = 350–490 nm, λ_{emiss} = 510 nm, slit widths = 2.5 nm).

HPLC Analysis of the Lactone and Carboxylate Forms of Irinotecan

Total irinotecan and relative proportions of the lactone and carboxylate species concentrations were determined using HPLC analysis as described by Chollet *et al.* (30), with modifications. Briefly, standard curves for the two species of irinotecan were generated by dissolving the drug in PBS pH 2.0 for the lactone species, or PBS pH 9.0 for the carboxylate species. The standards were incubated at room temperature for 1 h to reach equilibrium and both standards and liposomes were solubilized in ice-cold methanol immediately prior to analysis. Separation was achieved using a Symmetry® C_{18} cartridge column (100 Å, particle size 5 μm; 3.9 × 150 mm I.D., Waters, Milford, MA) with a run time of 9 min at a flow rate of 1.0 ml/min, and maintained at 35°C. A two-solvent mobile phase consisted of mobile phase A (3% triethlyamine, pH 6.4 adjusted with glacial acetic acid) and mobile phase B (100% acetonitrile), with the elution gradient containing a mixture of 78% A:22% B. For sample analysis, 80 μl of each sample and standard were loaded into 1 ml HPLC sample vials (Waters) with 200 μl inserts (Chromatographic Specialties Inc., Brockville, ON) and 5 μl was l was injected into the column. Irinotecan was quantified using an HPLC system equipped with a Waters Model 717 plus autosampler (set to 4°C), a Model 600E pump and controller and a Model 470 scanning fluorescence detector (Waters) set at an excitation wavelength of 362 nm and an emission wavelength of 425 nm. Data were acquired and processed with the Millenium32® chromatography manager (Version 3.20).

TLC Analysis of the Lactone and Carboxylate Forms of Irinotecan

Irinotecan controls were prepared by dissolving the drug in PBS buffer at the desired pH and incubating at room temperature for 1 h to allow equilibrium between the lactone and carboxy forms. Controls and liposomal samples were solubilized in $CHCl_3$/MeOH (1:1 v/v) and spotted on a TLC plate. The lactone and carboxy forms of the drug were separated by exposing the TLC plate initially to a mobile phase of $CHCl_3$/MeOH/acetone (9:3:1 v/v/v) for 30 min. The plate was then dried and subject to a further 4 h exposure to a second mobile phase comprising butanol/acetic acid/water/acetone (4:2:1:1 v/v/v/v). The drug species were visualized under UV light.

Cryo-Transmission Electron Microscopy

Radioactive-free DSPC/chol liposomes were prepared in unbuffered 300 mM $CuSO_4$, or 300 mM $CuSO_4$ buffered to pH 7.5 as described above and subsequently loaded with irinotecan at a drug-to-lipid ratio (mol/mol) of 0.2. In a chamber of controlled temperature (25°C) and humidity, 1–2 μl of liposome sample was deposited on copper grids coated with a holey cellulose acetate butyrate polymer. Excess liquid was thereafter blotted away with filter paper. The samples were quickly vitrified by plunging into liquid ethane and transferred to liquid nitrogen keeping the sample below 108 K, therefore minimizing sample pertubation and the formation of ice crystals. The grid was transferred to a Zeiss EM902 transmission electron microscope where observations were made in a zero-loss bright-field mode and an accelerating voltage = 80 kV.

RESULTS

Liposome Encapsulation of Irinotecan as a Function of Entrapped Metal Solution and Interior pH

DSPC/chol (55:45 mol%) liposomes formulated with internal unbuffered $CuSO_4$, $ZnSO_4$, $MnSO_4$ or $CoSO_4$ solutions (pH < 6) were exchanged into a pH 7.5 buffer prior to incubation with irinotecan at a drug-to-lipid ratio of 0.2:1 (mol/mol). As summarized in Fig. 2A, the liposome preparations formulated with 300 mM $CuSO_4$ (open circle), or 300 mM $ZnSO_4$ (square) exhibited >98% irinotecan loading after a 10 min incubation at 50°C. Those formulations with encapsulated solutions of $MnSO_4$ (triangle) or $CoSO_4$ (inverted triangle) did not mediate active encapsulation of irinotecan under the conditions described.

The formation of transition metal coordination complexes may be pH dependent, therefore several studies were initiated to assess how changes in pH (inside and outside) influenced irinotecan encapsulation. In the first study, liposome formulations were prepared with buffered internal transition metal solutions adjusted to pH 7.5 (300 mM $CuSO_4$ or $MnSO_4$, 20 mM HEPES, pH adjusted with TEA; stable for 24 h at room temperature). The external buffer was exchanged as described above, to create a metal ion gradient. Irinotecan loading into liposomes under these conditions, shown in Fig. 2B, indicate that Cu (open circle) effectively mediated the encapsulation of irinotecan at a rate and extent comparable to that shown in Fig. 2A. This demonstrated that active loading in the presence of Cu occurred independent of an initial imposed pH gradient. The liposomes prepared in the buffered Mn (pH 7.5) did not mediate any significant drug accumulation (Fig. 2B; triangle).

The drug loading characteristics of $ZnSO_4$ or $CoSO_4$ buffered to pH 7.5 could not be determined due to the instability of these salt solutions. Therefore, an alternative approach was taken to equilibrate the interior pH to the external pH while maintaining the metal ion gradient. Nigericin, a monovalent cation/proton ionophore that can exchange a proton from the liposome interior for one Na^+ ion from the exterior buffer has been demonstrated to collapse transmembrane pH gradients (20,31). The results in Fig. 2C shows that irinotecan loading into liposomes prepared with internal unbuffered solutions of $CuSO_4$ (open circle), $ZnSO_4$ (square), $MnSO_4$ (triangle) or $CoSO_4$ (inverted triangle) did not occur when the liposomes were incubated with nigericin prior to the addition of irinotecan. These results are in contrast to the data shown in Fig. 2B, where drug loading was achieved under conditions designed to obviate any transmembrane pH by buffering the interior $CuSO_4$ pH to 7.5. These data would suggest that a pH gradient may still exist under the conditions used to facilitate irinotecan uptake into liposome formulations prepared with buffered internal transition metal solutions adjusted to pH 7.5 (300 mM $CuSO_4$ or $MnSO_4$, 20 mM HEPES, pH adjusted with TEA).

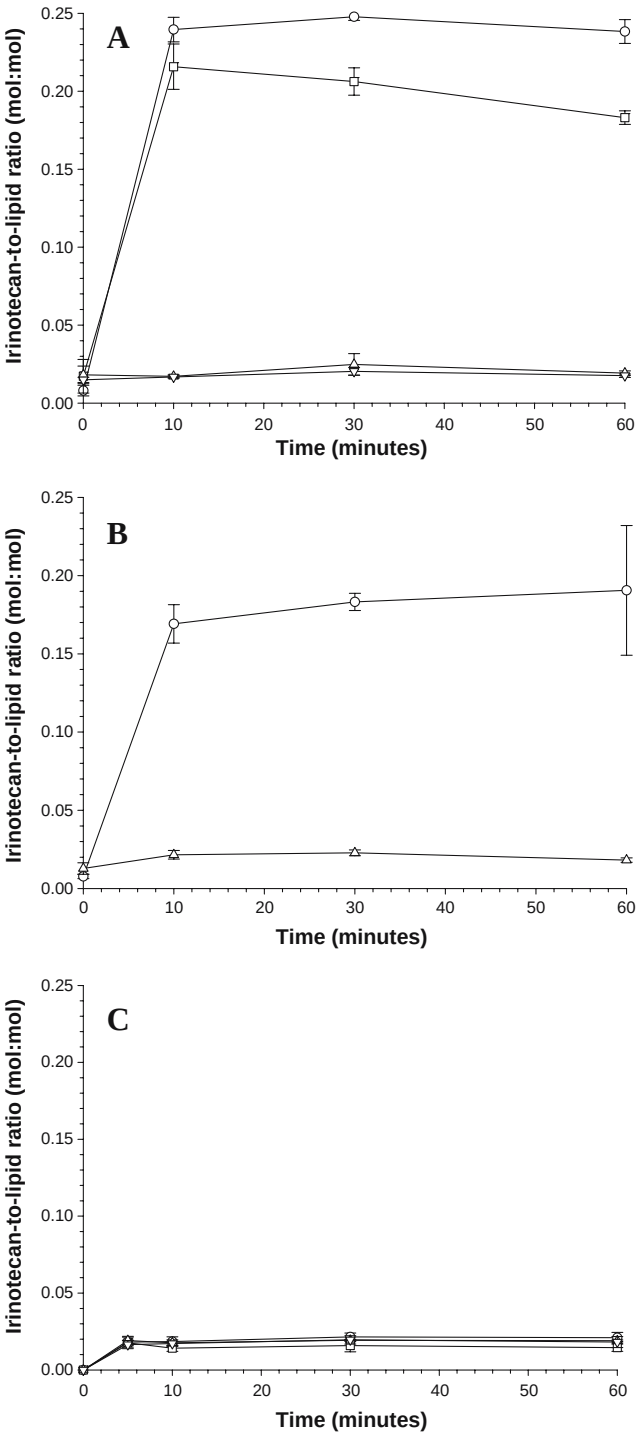

Fig. 2. DSPC/Chol liposome (55:45 mol%) encapsulation of irinotecan mediated by entrapped transition metal ion solutions. (A) Unbuffered solutions: 300 mM $CuSO_4$ (*open circle*), 300 mM $ZnSO_4$ (*square*), 300 mM $MnSO_4$ (*triangle*) and 300 mM $CoSO_4$ (*inverted triangle*); (B) solutions buffered at pH 7.5: 300 mM $CuSO_4$, 20 mM HEPES, TEA pH 7.5 (*open circle*) and 300 mM $MnSO_4$, 20 mM HEPES, TEA pH 7.5 (*triangle*); (C) unbuffered solutions + nigericin: 300 mM $CuSO_4$ (*open circle*), 300 mM $ZnSO_4$ (*square*), 300 mM $MnSO_4$ (*triangle*) and 300 mM $CoSO_4$ (*inverted triangle*). The ionophore nigericin was added to collapse any transmembrane pH gradient prior to incubation with irinotecan. The external buffers were exchanged with SHE pH 7.5 (300 mM sucrose, 20 mM HEPES, 15 mM EDTA pH 7.5) or, in the presence of nigericin, HBS pH 7.5 (20 mM HEPES, 150 mM NaCl pH 7.5). Liposomes (5 mM) were incubated with irinotecan at a drug-to-lipid ratio (mol/mol) of 0.2 at 50°C. At specified time points, the encapsulation efficiency (irinotecan-to-lipid ratio, mol/mol) was determined as described in "Materials and Methods". Data points represent the mean ± SD of at least three independent experiments.

The Influence of Interior and Exterior Liposome pH on the Efficiency of Irinotecan Encapsulation by Copper-Containing Liposomes

The data presented in Fig. 3 was obtained using liposomes prepared in buffered $CuSO_4$ (pH 7.5) that were exchanged into external 10 mM MES/10 mM HEPES/150 mM NaCl (MES) solutions adjusted to pHs ranging from pH 6.0 to pH 7.5. The external pH appeared to govern the rate and extent of irinotecan loading suggesting the ionization status of this weakly basic drug was dictating uptake rates. This is most evident at an incubation temperature of 50°C (Fig. 3A). If the MES buffer is at pH 7.0 (triangle) or pH 7.5 (inverted triangle), the efficiency of drug accumulation is >98% within 10 min. Conversely, at pH 6.0 (open circle) and pH 6.5 (square) there is a marked decrease in the rate of drug loading resulting in a lower measured irinotecan-to-lipid ratio after 30 min incubation. However, irinotecan accumulation does not appear to have reached a plateau, particularly at pH 6.0, suggesting that an increased incubation period could see loading efficiencies comparable to those observed at pH ≥ 7.0. This conclusion is supported by the results in Fig. 3B where the rate of drug loading was increased by raising the incubation temperature to 60°C. At this temperature all formulations exhibited >98% loading within 10 min.

The experiment summarized in Fig. 4 was designed to assess the influence of initial internal pH on irinotecan

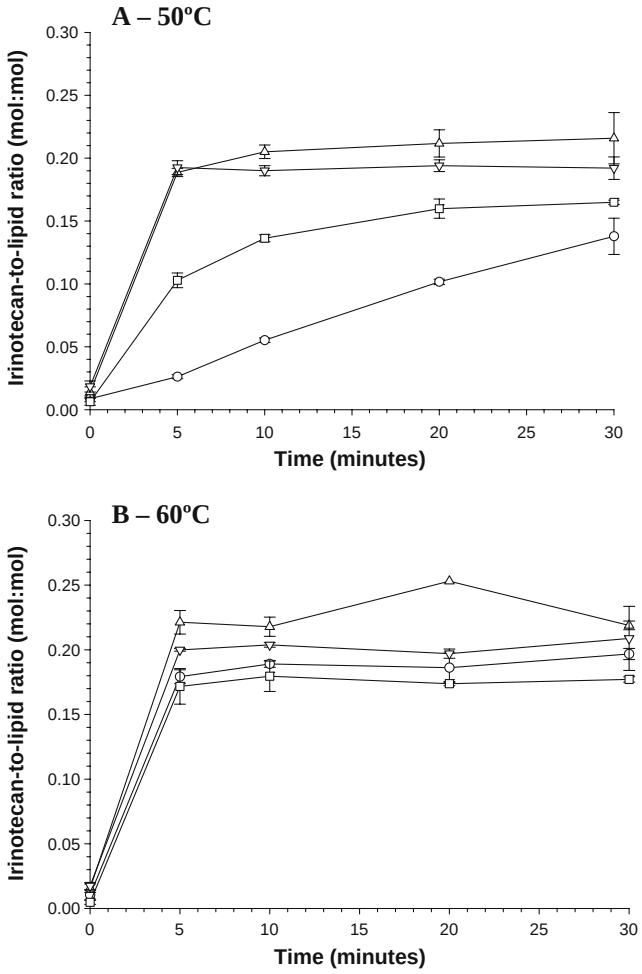

Fig. 3. DSPC/Chol liposome (55:45 mol%) encapsulation of irinotecan mediated by entrapped 300 mM $CuSO_4$ buffered at pH 7.5: The influence of incubation temperature and exterior 10 mM MES, 10 mM HEPES, 150 mM NaCl (MES) buffer pH on encapsulation efficiency. (A) 50°C: liposomes (5 mM) were incubated with irinotecan at a drug-to-lipid ratio (mol/mol) of 0.2 at 50°C in the presence of MES pH 6.0 (*open circle*), pH 6.5 (*square*), pH 7.0 (*triangle*) and pH 7.5 (*inverted triangle*). (B) 60°C: experimental conditions as described for (A) except the incubation temperature was increased to 60°C. At specified time points, the encapsulation efficiency [irinotecan-to-lipid ratio (mol/mol)] was determined as described in "Materials and Methods". Data points represent the mean ± SD of at least three independent experiments.

loading. The liposomes were prepared in $CuSO_4$ buffers (300 mM $CuSO_4$, 20 mM HEPES) adjusted to pH 6.0, pH 6.5, pH 7.0 or pH 7.5 with TEA. The resulting liposomes were then exchanged into a pH 7.5 buffer as described above. When evaluating loading at 50°C (Fig. 4A), the rate and extent of irinotecan accumulation was comparable when the internal pH was 6.0 (open circle), 6.5 (square) and 7.5 (inverted triangle). Unexpectedly, the rate of drug loading was reduced at pH 7.0 and only 75% of the added irinotecan became associated with these liposomes. Increasing the incubation temperature to 60°C had no effect on the drug loading characteristics for these formulations (Fig. 4B). The anomaly in irinotecan loading may be a reflection of the instability of the 300 mM $CuSO_4$/20 mM HEPES buffer which exhibited a tendency to precipitate at pH 7.0.

The final experiment was designed to assess the role of pH and pH gradients in irinotecan loading into $CuSO_4$-containing liposomes and evaluated the drug uptake when the initial pH of the interior Cu buffer and the exterior buffer were matched. The results, summarized in Fig. 5, indicated that the liposome preparations at pH 6.5 (square) and pH 7.5 (inverted triangle) displayed optimal irinotecan loading characteristics under these conditions. Consistent with the results shown in Fig. 3, the rate of irinotecan accumulation at pH 6.0 (open circle) at 50°C was reduced (Fig. 5A) and maximal drug loading efficiencies were not achieved. When the rate of loading was increased by increasing the incubation temperature to 60°C (Fig. 5B) loading efficiencies of >98%

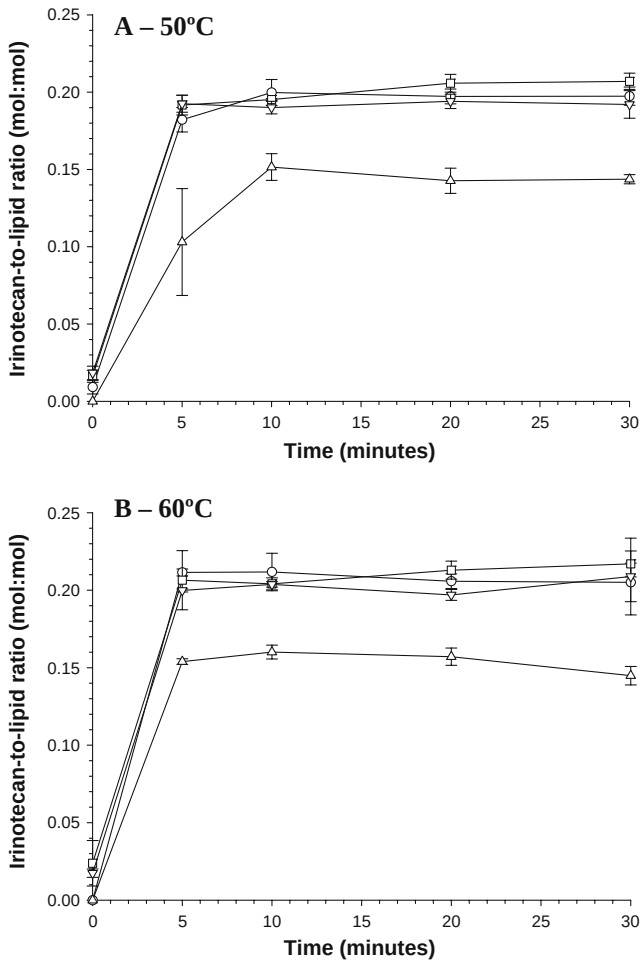

Fig. 4. DSPC/Chol liposome (55:45 mol%) encapsulation of irinotecan mediated by entrapped buffered $CuSO_4$ solutions: The influence of $CuSO_4$ pH and incubation temperature on encapsulation efficiency. Liposomes were formulated with internal 300 mM $CuSO_4$, 20 mM HEPES solutions buffered with TEA to: pH 6.0 (*open circle*), pH 6.5 (*square*), pH 7.0 (*triangle*) and pH 7.5 (*inverted triangle*). The external buffer was exchanged to SHE pH 7.5 (300 mM sucrose, 20 mM HEPES, 15 mM EDTA pH 7.5). (A) 50°C: liposomes (5 mM) were incubated with irinotecan at a drug-to-lipid ratio (mol/mol) of 0.2 at 50°C; (B) 60°C: experimental conditions as described for (A) except the incubation temperature was increased to 60°C. At specified time points, the encapsulation efficiency (irinotecan-to-lipid ratio, mol/mol) was determined as described in "Materials and Methods". Data points represent the mean ± SD of at least three independent experiments.

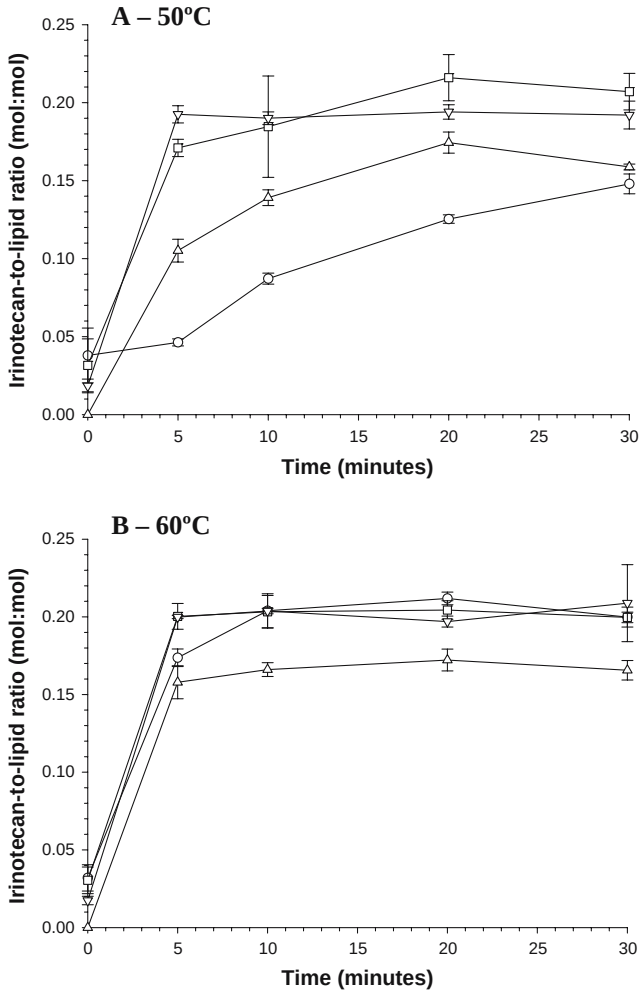

Fig. 5. DSPC/Chol liposome (55:45 mol%) encapsulation of irinotecan mediated by entrapped buffered 300 mM $CuSO_4$ solutions: the influence of matched internal and external buffer pH, and incubation temperature on encapsulation efficiency. Loading conditions tested were: $CuSO_4$/MES pH 6.0 (*open circle*), $CuSO_4$/MES pH 6.5 (*square*), $CuSO_4$/MES pH 7.0 (*triangle*) and $CuSO_4$/MES pH 7.5 (*inverted triangle*). (A) 50°C: liposomes (5 mM) were incubated with irinotecan at a drug-to-lipid ratio (mol/mol) of 0.2 at 50°C. (B) 60°C: experimental conditions as described for (A) except the incubation temperature was increased to 60°C. At specified time points, the encapsulation efficiency (irinotecan-to-lipid ratio, mol/mol) was determined as described in "Materials and Methods". Data points represent the mean ± SD of at least three independent experiments.

were observed within 10 min. Similar to the results shown in Fig. 3, the instability of the $CuSO_4$ buffer at pH 7.0 (triangle) resulted in aberrant drug loading behavior.

Assessment of the Interior pH of Copper-Containing Liposomes Following Irinotecan Encapsulation

To gain a better understanding of how irinotecan encapsulation affected the internal pH of liposomes with entrapped unbuffered $CuSO_4$, HPTS a pH-sensitive dye was used as an internal pH probe. This anionic fluorophore is water-soluble and membrane-impermeant and therefore, can be trapped in the liposome interior (32). Figure 6 shows the fluorescence emission intensity of HPTS recorded at 510 nm over the excitation wavelength range 400–500 nm. Liposomes formulated with entrapped unbuffered $CuSO_4$ (pH 3.5) and HPTS (X) have an emission maximum at 405 nm (thin arrow) which is characteristic of an acidic environment. Following irinotecan encapsulation into these liposomes (open circle) there is a shift in the major HPTS emission peak to $\lambda_{excit} = 450$ nm (thick arrow). This behavior is associated with a basic environment. Irinotecan-loaded liposomes in the absence of HPTS (filled square) had no detectable fluorescence at $\lambda_{excit} = 450$ nm (thick arrow). These results suggest there was partial/complete collapse of the initial pH gradient following irinotecan accumulation in liposomes prepared in unbuffered $CuSO_4$. Attempts to estimate the internal pH of buffered Cu liposomes using HPTS as a pH probe were unsuccessful due to interference of the assay by the Cu and/or irinotecan (data not shown).

The Irinotecan Loading Capacity and Cryo-TEM Analysis of Copper-Containing Liposomes

Liposomes formulated with unbuffered 300 mM $CuSO_4$, or buffered 300 mM $CuSO_4$ pH 7.5 were further characterized by evaluating the efficiency of irinotecan loading at 50°C as a function of increasing initial drug-to-lipid ratios (Fig. 7; 0.1 (open circle), 0.2 (square), 0.4 (triangle), 0.6 (inverted triangle) (mol/mol)). Regardless of whether the liposomes were prepared in unbuffered $CuSO_4$ solution (Fig. 7A) or buffered $CuSO_4$ solutions at pH 7.5 (Fig. 7B), the irinotecan loading attributes were comparable. At drug-to-lipid ratios

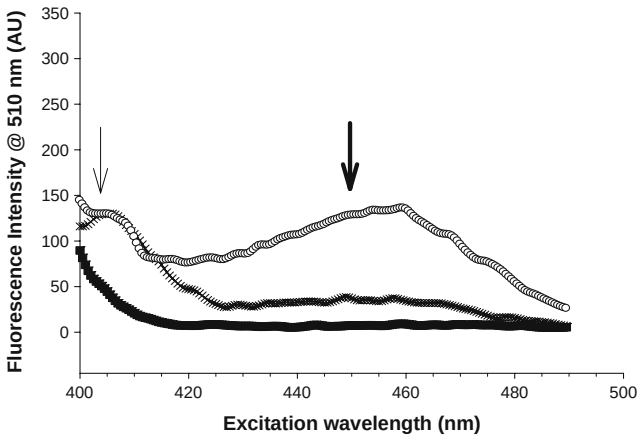

Fig. 6. Assessment of the internal pH of liposomes formulated with unbuffered 300 mM $CuSO_4$ before and after irinotecan encapsulation. DSPC/Chol (55:45 mol%) liposomes were formulated with (1) unbuffered 300 mM $CuSO_4$ + HPTS (12.5 mM) (X); (2) unbuffered 300 mM $CuSO_4$ loaded with irinotecan at a drug-to-lipid ratio (mol/mol) of 0.2 at 50°C (*filled square*); and (3) unbuffered 300 mM $CuSO_4$ + HPTS (12.5 mM) loaded with irinotecan at a drug-to-lipid ratio (mol/mol) of 0.2 at 50°C (*open circle*). The presence of HPTS did not impair the irinotecan encapsulation efficiency of the liposomes. Fluorescence emission at 510 nm was recorded as a function of excitation wavelength (400–500 nm). In acidic conditions HPTS has a maximum fluorescence emission ($\lambda = 510$ nm) at $\lambda_{excit} = 405$ nm (see *thin arrow*). In a more basic environment the fluorescence emission intensity ($\lambda = 510$ nm) increases at $\lambda_{excit} = 450$ nm (see *thick arrow*).

(mol/mol) of 0.1 (open triangle) and 0.2 (square), >98% of irinotecan was encapsulated within the liposomes after a 10-minute incubation period. As the drug-to-lipid ratio increased beyond 0.2, maximum loading was still achieved within 10 min; however, the efficiency of irinotecan loading decreased. A maximum encapsulation efficiency of 58% was obtained when the initial drug-to-lipid ratio (mol/mol) was 0.6 (inverted triangle). The extent of drug loading was comparable for both liposomal formulations and the maximum level of uptake achievable in these formulations appeared to be between 0.3 and 0.35 mole irinotecan per mole total lipid (Fig. 7).

The data presented thus far indicates that the irinotecan loading characteristics of Cu-based liposomes are comparable regardless of whether an initial transmembrane pH gradient exists or not. To determine whether there were any morphological differences between the two formulations, the liposomes were analyzed by cryo-TEM. In the absence of irinotecan, the "empty" liposomes prepared with 300 mM $CuSO_4$, 20 mM HEPES, TEA pH 7.5 did not appear to have any visible metal precipitates (Fig. 7C). In contrast, following irinotecan accumulation into these liposomes a dark spot (arrows) was consistently observed (Fig. 7D). Irinotecan encapsulation into liposomes prepared in unbuffered $CuSO_4$ also resulted in the appearance of intravesicular electron-dense regions (arrows, Fig. 7E).

The Influence of the Lactone Ring Conformation on Irinotecan Encapsulation with Copper-Containing Liposomes

The therapeutic activity of irinotecan is dependent on the integrity of the lactone ring and this has been attributed, in part, to enhanced passive diffusion through cell membranes (33). The rate of irinotecan diffusion across the liposomal membrane and subsequent entrapment in the Cu environment of the aqueous core can be investigated as a function of lactone ring status. The carboxylate (ring open) form of irinotecan can be generated by incubating the drug in a borate buffer at pH 9.0 for 1 h at room temperature. HPLC analysis (not shown) confirmed that >99.9% of the irinotecan was in the carboxylate form under these conditions. The carboxylate form of irinotecan was mixed with buffer and the pH was adjusted to pH 7.5 immediately prior to addition to

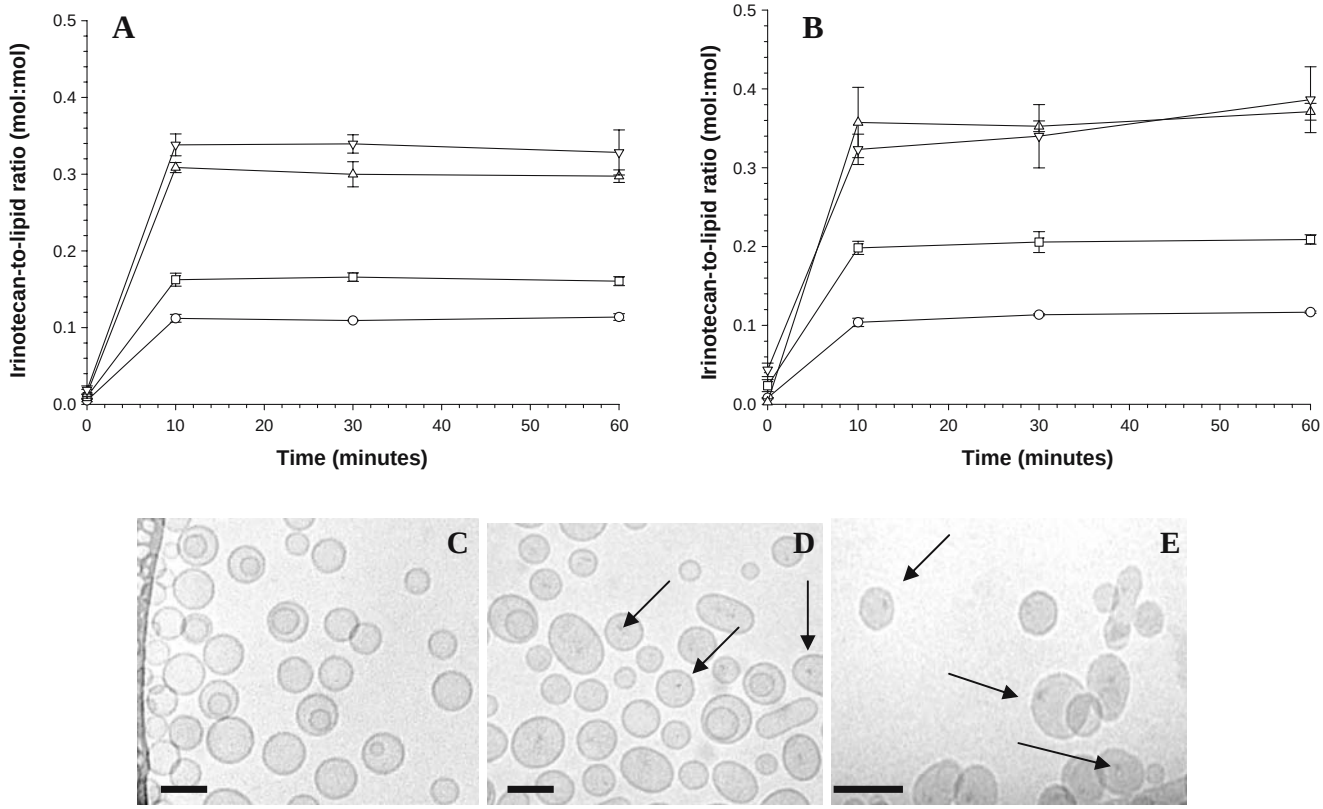

Fig. 7. Irinotecan encapsulation into DSPC/Chol liposomes (55:45 mol%) prepared with: (A) unbuffered 300 mM $CuSO_4$ (initial unbuffered pH ~ 3.5) and, (B) 300 mM $CuSO_4$, 20 mM HEPES, buffered with TEA to pH 7.5. The external solutions were exchanged with SHE pH 7.5 (300 mM sucrose, 20 mM HEPES, 15 mM EDTA pH 7.5). The irinotecan loading efficiency and capacity was determined at 50°C for initial drug-to-lipid (mol/mol) ratios—0.1:1 (*open circle*), 0.2:1 (*square*), 0.4:1 (*triangle*), 0.6:1 (*inverted triangle*). At specified time points, the encapsulation efficiency (irinotecan-to-lipid ratio, mol) was determined as described in "Materials and Methods". Data points represent the mean ± SD of at least three independent experiments. (C) Cryo-TEM representation of "empty" DSPC/Chol liposomes formulated with 300 mM $CuSO_4$, 20 mM HEPES, buffered with TEA to pH 7.5. (D) A representative cryo-TEM image of DSPC/Chol liposomes formulated with 300 mM $CuSO_4$, 20 mM HEPES, buffered with TEA to pH 7.5 following irinotecan encapsulation. The *arrows* indicate dark intravesicular spots. (E) A representative cryo-TEM image of unbuffered 300 mM $CuSO_4$ DSPC/Chol liposomes loaded with irinotecan. The *arrows* indicate dark intravesicular spots. The bars shown in (C), (D) and (E) = 100 nm.

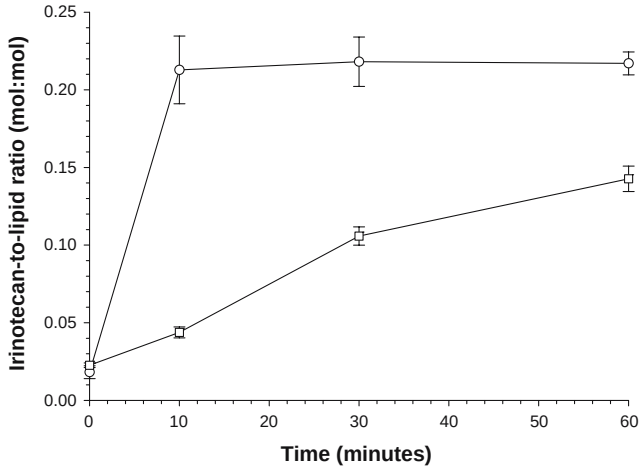

Fig. 8. The rate and efficiency of irinotecan encapsulation by DSPC/Chol (55:45 mol%) liposomes as a function of the integrity of the lactone ring. Irinotecan was pre-incubated for 1 h at room temperature in the presence (*square*), or absence (*open circle*), of borate buffer pH 10.0. The liposome internal buffer comprised 300 mM $CuSO_4$, 20 mM HEPES, buffered with TEA to pH 7.5. The external buffer was exchanged with SHE pH 7.5. Immediately after addition of the pre-incubated drug to the liposome suspension at 50°C [drug-to-lipid (mol/mol) ratio of 0.2], the pH was adjusted to pH 7.5. At specified time points, the encapsulation efficiency (irinotecan-to-lipid ratio, mol/mol) was determined as described in "Materials and Methods". Data points represent the mean ± SD of at least three independent experiments.

liposomes (initial drug-to-lipid ratio of 0.2, mol/mol). The results, summarized in Fig. 8, indicate that the carboxylate form (square) is not efficiently loaded into the copper-containing liposomes. In the absence of a pH 9.0 pre-incubation, irinotecan accumulation efficiency was >98% after a 10 min incubation period (open circle). In contrast, if irinotecan was preincubated at pH 9.0 prior to addition to the liposomes only 20% uptake was observed after a 10 min incubation and <40% uptake was observed at 30 min. It should be noted that when the preincubated drug was added to the liposomes the pH was adjusted to pH 7.5. Under these conditions, the carboxy form would be expected to undergo lactonization to reach equilibrium between the two forms of the drug. Lactonization is a much slower process than hydrolysis (34) and could explain the gradual increases in the irinotecan-to-lipid ratio over time. Therefore, extending the incubation period could facilitate maximum irinotecan loading by: (a) allowing the conversion of carboxy to lactone, and (b) by sequestering the lactone form of irinotecan in the liposome interior thereby forcing the equilibrium to the right (see Figure 1).

Thin-Layer Chromatography (TLC) and High-Performance Liquid Chromatography (HPLC) Analysis of Irinotecan Encapsulated in Copper-Containing Liposomes

TLC and HPLC were used to qualitatively and quantitatively assess irinotecan following accumulation into liposomes prepared using unbuffered 300 mM $CuSO_4$ or buffered 300 mM $CuSO_4$/20 mM HEPES/TEA (pH 7.5). A representative TLC plate is shown in Fig. 9. Irinotecan standards were included to provide a qualitative measure of the proportion of the lactone and carboxylate forms of the drug in buffers of known pH (Fig. 9). For example, at pH 2.0, irinotecan is exclusively present as the closed ring lactone form. At pH 6.0, it is evident that a proportion of the drug now exists as the open-ring carboxylate form. Subsequent increases in pH shift the equilibrium towards the carboxylate, such that at pH 10.0 the drug exists entirely in its open-ring state (Fig. 9, last lane). Irinotecan encapsulated in liposomes

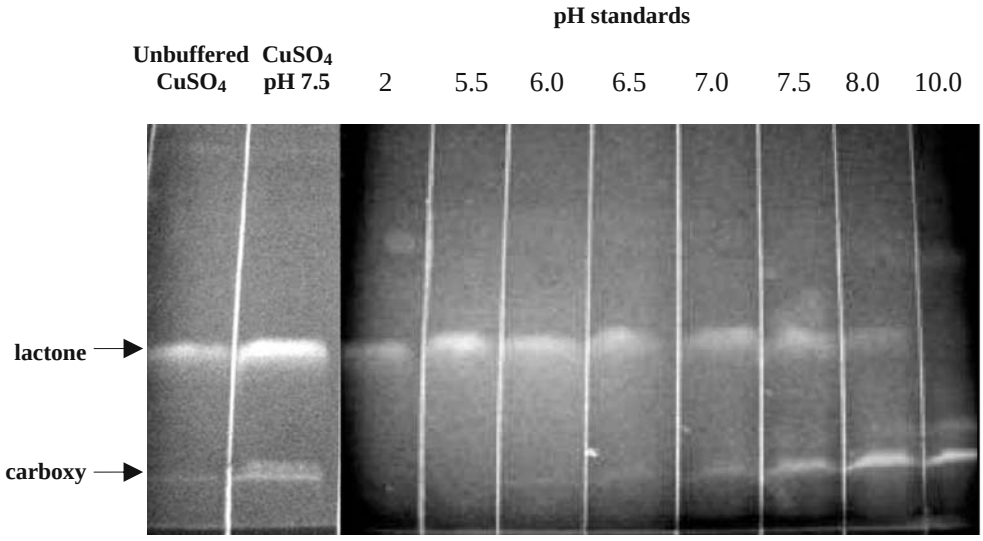

Fig. 9. Thin-layer chromatography (TLC) analysis of the ring status of irinotecan encapsulated in Cu-containing liposomes. Irinotecan controls and liposomal samples were solubilized in $CHCl_3$/MeOH (1:1 v/v) and spotted on a TLC plate. The lactone and carboxy forms of the drug were separated by exposing the TLC plate initially to a mobile phase of $CHCl_3$/MeOH/acetone (9:3:1 v/v/v) followed by a mobile phase of butanol/acetic acid/water/acetone (4:2:1:1 v/v/v/v). The drug was visualized under UV light.

prepared in unbuffered $CuSO_4$ was found to exist predominately as the active lactone form (Fig. 9, first lane). HPLC analysis determined that the lactone fraction accounted for 96% of the total drug (data not shown). Similarly, when irinotecan was encapsulated in liposomes comprising an interior Cu buffer pH 7.5, the majority of entrapped drug was in the lactone form. However, the proportion of carboxylate present appeared to be greater than that seen for the unbuffered $CuSO_4$ formulation (Fig. 9, second lane). This was confirmed by subsequent HPLC analysis which indicated that 83% of the total drug was in the closed ring conformation. It should be noted that these observations are consistent for both formulations for at least 7 days after drug loading.

DISCUSSION

Kuwahara described the formation of a 1:1 Cu/CPT complex where the putative Cu interaction with the oxygen atoms of the lactone ring (26) was later supported by EPR measurements (35). This observation prompted us to investigate whether the transition metal ions Cu, Zn, Mn and Co could: (a) promote the efficient encapsulation of irinotecan into preformed liposomes, and (b) stabilize the drug it its lactone form. Figure 2A demonstrated that only liposomes with entrapped unbuffered solutions of $CuSO_4$ or $ZnSO_4$ efficiently loaded irinotecan (>98% encapsulation). The Cu formulation maintained a stable irinotecan-to-lipid ratio for up to 60 min. In contrast, the Zn preparation appeared to be slowly releasing associated drug over time indicating that this formulation may not be as stable as its Cu counterpart. Under these drug-loading conditions, significant irinotecan accumulation was not observed for liposomes prepared in $MnSO_4$ or $CoSO_4$. This result led to two conclusions: (1) the choice of the encapsulated metal is a critical factor in facilitating drug loading and (2) since all unbuffered metal solutions were acid (pH < 5), the initial transmembrane pH gradient present was not sufficient in itself to promote irinotecan loading.

The ionophore nigericin was used in an attempt to investigate the capacity for the unbuffered metal-containing liposomes to encapsulate irinotecan in the absence of an initial transmembrane pH gradient (Fig. 2C). Nigericin exchanges a proton from the liposome interior with a Na^+ from the exterior buffered saline resulting in a more basic metal solution with a concomitant abolition of the pH gradient. Under these conditions, there was no significant uptake of irinotecan mediated by any of the divalent metal formulations tested. Interestingly, we have recently demonstrated that another water-soluble camptothecin analogue, topotecan, was efficiently encapsulated by Cu-containing DSPC/Chol (55:45 mol%) liposomes in the presence of nigericin (36). In contrast, when liposomes were prepared with encapsulated Cu solutions at a pH comparable to the exterior pH (e.g., no initial transmembrane pH gradient) by encapsulating pH 7.5 buffered metal solutions resulted in a Cu-mediated irinotecan-loading profile that was comparable to the unbuffered Cu formulation (Fig. 2B). For this reason we can suggest that the presence of an initial transmembrane pH gradient is not required for Cu-mediated drug loading, while also suggesting on the basis of the studies with nigericin that development of a transient pH gradient may be needed. It should be noted that the divalent metal ion solutions are not particularly stable at higher pH and we were only able to prepare $CuSO_4$ and $MnSO_4$ buffers at pH 7.5 with TEA. These formulations may not exhibit a pH gradient when first exchanged into the buffered saline solution that is required to create the copper ion gradient; however, this method generates a TEA gradient (high TEA concentration inside). Movement of TEA from the inside of the liposome to the outside may cause a decrease in the interior pH; analogous to pH gradient loading methods that rely on encapsulated ammonium sulfate (19). The pH gradient generated in response to TEA movement from the liposomes would not be stable in the presence of nigericin. This mechanism may explain why Cu-containing liposomes were unable to mediate effective irinotecan encapsulation in the presence of nigericin; but it does not explain why nigericin did not influence loading of topotecan under comparable conditions (36). These differences may by due to the chemical nature (e.g., hydrophobicity) of the two camptothecins and/or the manner in which the metals complex the drug.

We have previously reported that Mn mediated the active loading of doxorubicin into preformed liposomes (21). It is evident, however, that Mn is unable to mediate irinotecan encapsulation (Fig. 2). Further, Zn was able to promote loading of irinotecan (Fig. 2A), but not topotecan (36). Therefore, both the nature of the metal and the presence of distinct complexing groups on each individual drug, even within the same class, will determine whether this method can be used to promote accumulation of drugs within a liposomal carrier. The drug metal interaction is exemplified by the cryo-TEM analysis that revealed the presence of electron-dense intravesicular spots for the irinotecan-loaded formulations prepared with copper-containing liposomes. In contrast, topotecan-loaded liposomes prepared using liposomes that contained unbuffered $CuSO_4$ adopted a "coffee bean" morphology comprising elongated liposomes with distinctive internal linear precipitates (36). The latter was comparable to precipitates seen for doxorubicin loaded liposomes (21).

A main objective of this study was to determine whether transition metals were capable of stabilizing the lactone form of irinotecan. HPLC and TLC analysis of irinotecan from drug loaded liposomes confirmed that the majority of the encapsulated drug is in its lactone form (Fig. 9). Since the carboxylate form of the drug appears to be less membrane permeable (see Fig. 8) it is likely that the lactone form of the drug is crossing the liposomal membrane. Subsequently, copper complexation effectively traps the drug inside the liposome. Lactone stabilization could be due to one of two reasons. First, copper complexation with irinotecan at the site of the lactone ring could stabilize the structure preventing hydrolysis at neutral pH. Alternatively, the interior pH of the liposome may be acidic (<6.5), thus stabilizing the drug in its lactone form.

In summary, this report describes a novel remote-loading procedure, where entrapped Cu^{2+} and Zn^{2+} ions mediate the rapid and stable accumulation of irinotecan within DSPC/chol liposomes. Focusing here on Cu^{2+} containing liposomes, it was shown that irinotecan loading occurred in the presence or absence of an initial transmembrane pH gradient. Ongoing studies in our laboratory are evaluating the anti-cancer efficacy of this preparation in pre-clinical models.

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