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Role of copper gluconate/triethanolamine in irinotecan encapsulation inside the liposomes

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Abstract

A novel method for encapsulating irinotecan into liposomes containing copper gluconate buffered to pH 7.0 with triethanolamine (TEA) has recently been developed. In the present study, the mechanism dictating drug encapsulation and retention inside those liposomes was investigated. Spectroscopic analyses revealed that irinotecan interacted with copper gluconate/TEA in solution. Fourier transformed infrared (FT-IR) spectroscopy indicated a strengthening of the hydrogen bonds involving the hydroxyl groups when solutions of irinotecan and copper gluconate/TEA are mixed at a 1:1 molar ratio. The intensity of the circular dichroism (CD) signal of copper gluconate/TEA increased in the presence of equimolar amounts of irinotecan. The addition of irinotecan to liposomes containing copper gluconate/TEA at 50 °C induced a shift of the absorption bands from 370 nm to 378 nm as well as a 60% quenching of the drug fluorescence at 440 nm suggesting the occurrence of irinotecan self association. Irinotecan encapsulation was found to be kinetically and stoichiometrically correlated with the release of TEA from the liposomes. The results suggested that the encapsulation of irinotecan was mediated by TEA in association with copper gluconate, leading to a final drug complex that is retained inside the liposomes. A neutral antiport exchange loading mechanism between irinotecan and TEA is proposed.

Keywords: Irinotecan; Spectroscopy; Copper gluconate; Triethanolamine; Liposomes

1. Introduction

Two primary techniques are routinely used for the encapsulation of drugs within liposome carriers. One method is passive encapsulation where liposomes are formed in the presence of the drug (Bally et al., 1988; Mayer et al., 1989). A second, more efficient, "active loading" method involves the formation of a transmembrane pH gradient through the use of citrate, ammonium sulfate or ionophore/divalent cation (Mayer et al., 1985; Huang et al., 1992; Boman et al., 1993; Haran et al., 1993; Cullis et al., 1997; Cheung et al., 1998; Drummond et al., 2006; Noble et al., 2006). The acidified liposomal interior causes the loading and retention of drugs with ionizable moieties such as amine groups (Madden et al., 1990; Cullis et al., 1991). This method allows for efficient drug encapsulation, generally greater than 80%, but also has certain disadvantages. For example, several clinical formulations of such liposomal drugs require the generation of the pH gradient just prior to drug loading due to gradient and/or drug instability (Conley et al., 1993; Gelmon et al., 1999). A second disadvantage is the potential hydrolysis of lipids at acidic pH, which can introduce liposome instability during long-term storage (Barenholz et al., 1993; Grit and Crommelin, 1993). Ideally, a loading method would allow for efficient encapsulation at a neutral pH to prevent drug and lipid degradation.

For more than 40 years, chemists have studied the interactions between transition metals and drugs (Foye, 1961). During this period, it has become apparent that the strength of metal binding is dependent on the metal species as well as the drug. If drugs could be encapsulated inside liposomes containing transition metals, then the strength of the interaction may be useful not only for achieving high efficiency encapsulation, but also for customizing drug release rates. Recently, we have described the ability of copper to effectively encapsulate the water-soluble drug irinotecan whereas minimal drug encapsulation occurred when manganese, nickel and cobalt were used (Ramsay et al., 2004; Tardi et al., in press). A loading method that allowed efficient drug encapsulation using copper at neutral

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pH without generation of a pH gradient was developed. Various copper salts were tested and liposomes containing copper gluconate, adjusted to neutral pH with triethanolamine (TEA), was found to be the most appropriate for drug loading (Tardi et al., in press). This novel method for drug encapsulation was used to develop CPX-1, a liposomal delivery system containing irinotecan and floxuridine in a fixed 1:1 molar ratio that coordinates the *in vivo* release of the two anticancer drugs (Batist et al., 2006). CPX-1 is in phase 2 clinical development as a treatment for colorectal cancer (Mayer et al., 2006).

This study investigates the basis by which copper gluconate/TEA encapsulates and retains irinotecan inside the liposomes. The original hypothesis was that the loading of irinotecan occurred through complexation to copper. For that reason, a study was carried out to establish any evidence of irinotecan binding to copper and identify the binding sites involved in this interaction. The study was first performed in solution using Fourier Transformed Infrared (FT-IR) and circular dichroism (CD) spectroscopy to avoid interferences from the liposomes. More insight into the state of irinotecan inside the liposomes was obtained by ultraviolet–visible (UV/vis) and fluorescence spectroscopy of the liposome encapsulated drug. Based on the results, a mechanism for irinotecan encapsulation and retention into the liposomes containing copper gluconate/TEA is proposed.

2. Materials and methods

2.1. Materials

Irinotecan was obtained from ScinoPharm Taiwan Ltd (Tainan, Taïwan). Copper gluconate was purchased from Purac (Lincolnshire, IL, USA). 1,2,-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC) and 1,2,-distearoyl-*sn*-glycero-3-phosphoglycerol sodium salt (DSPG) were purchased from Lipoid (Newark, NJ, USA). Cholesterol (Chol) was obtained from Solvay (Houston, TX, USA). [^{3}H]-cholesteryl hexadecyl ether (^{3}H-CHE) was obtained from Perkin-Elmer (Shelton, CT, USA). All other chemicals were obtained from Sigma–Aldrich Corp (St Louis, MO, USA).

2.2. Irinotecan and copper gluconate/TEA solution preparation

The solutions of irinotecan were made by dissolving the drug either in water at 50 °C or in sucrose phosphate buffer (300 mM sucrose, 40 mM phosphate, pH 7.0) at room temperature. When necessary, the pH of the solution was adjusted to the desired value using sodium hydroxide (NaOH). The final concentration of irinotecan was 15 mM. The 100 mM copper gluconate buffer was prepared by dissolving the copper gluconate powder in water at room temperature and adjusting the pH to 7.0 using NaOH or TEA. The final concentration of TEA required to buffer the solution of copper gluconate to pH 7.0 was 180 mM. For solutions of copper gluconate with 90 mM and 270 mM TEA, the pH was brought to 7.0 with NaOH and hydrochloric acid (HCl), respectively. The solution of 10 mM sodium gluconate/180 mM TEA was made by dissolving the sodium gluconate in water, adding TEA and finally adjusting the pH to 7.0 with HCl. Mixtures of irinotecan and copper gluconate/TEA were made to obtain a drug:metal molar ratio of 1:1. Further addition of irinotecan to copper gluconate/TEA at higher drug:metal ratios caused the formation of a precipitate in the solution. The precipitate was isolated by centrifugation for 15 min at 13,400 × g and was solubilized with 2 mM ethylenediaminetetraacetic acid (EDTA) in water.

2.3. Liposome preparation

The phospholipids and cholesterol were dissolved in chloroform/methanol/water (95/4/1, v/v/v) at a molar ratio of 7:2:1 for DSPC:DSPG:Chol. The lipids were labeled with trace amounts (~0.005 μCi/μmol lipid) of ^{3}H-cholesteryl hexadecyl ether, a non-exchangeable, non-metabolizable lipid marker to allow liposome quantitation by scintillation counting. The solvent was evaporated under a stream of nitrogen and dried under vacuum for at least 4 h. The sample was hydrated with 100 mM copper gluconate/180 mM TEA, pH 7.0 or 150 mM TEA/95 mM phosphate, pH 7.0 or 10 mM sodium gluconate/180 mM TEA, pH 7.0 to obtain a final lipid concentration of 50 mg/ml. The resulting lipid solution was extruded 10 times at 70 °C through two polycarbonate filters with 0.1 μm pores diameters at moderate pressure using a liposome extruder (Lipex Inc., Vancouver, BC). The external buffer was finally exchanged with a 300 mM sucrose/40 mM phosphate/10 mM EDTA, pH 7.0 buffer by cross-flow filtration.

2.4. Drug encapsulation into radiolabeled liposomes

The liposomes and the irinotecan solution were made as described above. The drug solution and the liposomes were incubated separately at 50 °C for approximately 5 min to equilibrate the temperature. The irinotecan solution and liposome dispersion were combined to obtain a 0.2:1 drug to lipid molar ratio; aliquots were removed at various time points and put on ice to stop the loading the process. Aliquots of 75 μl were applied to a Sephadex G-50 spin column. The columns were prepared by adding glass wool to a 1 ml syringe and Sephadex G-50 beads hydrated in sucrose phosphate buffer (300 mM sucrose, 40 mM phosphate, pH 7.0). The columns were packed by spinning at 290 × *g* for 1 min. Following addition of the sample to the column, the liposome fraction was collected in the void volume by centrifuging at 515 × *g* for 1 min. Aliquots of the spin column eluant and the pre-column solution were taken and analyzed by liquid scintillation counting to determine the lipid concentration at each time point. The irinotecan concentration in each liposomal fraction was determined using a UV-based assay. Briefly, a 100 μl aliquot of each liposomal sample (or smaller volume adjusted to 100 μl with distilled water) was solubilized in 100 μl of 10% Triton X-100 plus 800 μl of 50 mM citrate/trisodium citrate, 15 mM EDTA, pH 5.5 and heated in boiling water until the cloud point was reached. The samples were cooled to ambient temperature. The absorbance at 370 nm was measured

and compared to a standard curve. The concentration of TEA was determined by high performance liquid chromatography (HPLC).

2.5. Triethanolamine quantitation using HPLC

To determine the concentration of TEA, the liposomes were disrupted with methanol then diluted in water. The sample was centrifuged for 30 min at 16,100 × *g*. An aliquot of the supernatant was analyzed on a Waters Alliance HPLC system equipped with a Phenomenex Luna C18(2) reverse phase column (4.6 × 150 mm, 5 μ) maintained at 30 °C and an evaporative light scattering detector (ELSD 2000, Alltech, IL, USA). The mobile phase composed of 2 mM nonafluoropentanoic acid and acetonitrile (90:10, v/v), was delivered isocratically at a flow rate of 1 ml/min. The injection volume was 20 μl. The concentration of TEA was calculated using a calibration curve. The detection and integration of chromatographic peaks were performed by Waters Empower software.

2.6. Cryogenic transmission electron microscopy

The cryogenic transmission electron microscopy (cryo EM) investigations were performed with a Zeiss EM 902A transmission electron microscope (Carl Zeiss NTS, Oberkochen, Germany). The instrument was operated at 80 kV and in zero loss bright-field mode. Digital images were recorded under low dose conditions with a BioVision Pro-SM Slow Scan CCD camera (Proscan GmbH, Scheuring, Germany) and analyzed with analySIS software (Soft Imaging System, GmbH, Münster, Germany). The procedure used for sample preparation and image recording is described in Almgren et al. (2000). Briefly, the samples were equilibrated at 25 °C and approximately 99% relative humidity within a climate chamber. A small drop (∼1 μl) of sample was deposited on a copper grid covered with a perforated polymer film covered with a thin carbon layer on both sides. Excess liquid was removed by means of blotting with a filter paper, leaving a thin film of the solution on the grid. Immediately after blotting, the sample was vitrified in liquid ethane, held just above its freezing point. Samples were kept below −165 °C and protected against atmospheric conditions during both transfer to the microscope and examination.

2.7. CD and FT-IR spectroscopy

FT-IR measurements were made at room temperature in transmission mode using a Nicolet Nexus 870 spectrometer (Nicolet Instrument, Madison, WI, USA) equipped with a liquid nitrogen-cooled mercury cadmium telluride detector. Spectra of dry films of irinotecan and irinotecan/copper gluconate mixtures were obtained by spreading 20 μl of the sample on a BaF_2 window (Wilmad Glass Co. Inc., Buena, NJ). The sample was dried with a stream of nitrogen and left overnight in a desiccator before recording the spectra. For each spectrum, 250 scans were co-added at a 4 cm^{-1} resolution, using a Happ-Genzel apodization. Data analysis was done using Grams AI software (Galactic Industries, Salem, NH, USA). The second derivative of the spectra was performed to determine the frequency of the components of unresolved bands.

Circular dichroism analyses were conducted using a JASCO J-810 spectropolarimeter, calibrated with a solution of 1% D-camphor-10-sulfonic acid in water. All spectra were recorded at 25 °C between 190 nm and 800 nm using a quartz cell with a 1 cm or a 0.2 cm path length. For each spectrum, 2 scans were accumulated at a scanning speed of 50 nm/min.

2.8. Fluorescence and UV/vis spectroscopy

Samples were prepared using the aliquots taken during the loading process, as described above, before applying to the Sephadex G-50 spin columns. The aliquots were diluted in sucrose phosphate buffer (300 mM sucrose, 40 mM phosphate, pH 7.0) to obtain a final irinotecan concentration of 6 μM. The same dispersions were used for both UV/vis and fluorescence measurements. The spectra of the liposomes alone were not subtracted from the spectra of the mixtures because the contribution of the liposomal signal to that of the drug was found to be negligible. UV/vis spectra were recorded with a Shimadzu 2401-PC spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD). Fluorescence spectra were recorded using either a Perkin-Elmer (model LS 50B, Perkin-Elmer Life and Analytical Sciences, Woodbridge, ON) or a Varian Cary Eclipse (Varian, Palo Alto, CA) spectrofluorometers. For fluorescence measurements, the excitation wavelength was set at 400 nm and the emission scans were obtained from 425 nm to 650 nm. The slits were set at 2.5 nm. Measurements were made at ambient temperature using a quartz cell with a 1 cm path length.

3. Results

3.1. Circular dichroism

The presence of chiral centers in the gluconate moieties allowed the use of circular dichroism to investigate possible interactions between irinotecan and copper gluconate/TEA. The CD spectrum of a 2.5 mM solution of copper gluconate buffered to 7.0 with 4.5 mM TEA exhibited a broad band centered at 630 nm whose intensity increased from 8 mdeg to 13 mdeg upon addition of 1 mol-equiv. of irinotecan to copper gluconate/TEA (Fig. 1).

Since irinotecan has a chiral center located on carbon 2 of the lactone ring (Fig. 2), we also investigated the possibility of characterizing its potential interaction with copper gluconate/TEA by examining changes in the drug CD band. At drug concentrations greater than 250 μM, the high absorption of irinotecan induced artifacts in the CD signal. Therefore, spectra were recorded using low concentrations of the drug. The CD spectrum of irinotecan at 250 μM exhibited two conservative CD signals in the UV region (not shown). Addition of copper gluconate/TEA (pH 7.0) to irinotecan at a 1:1 molar ratio did not induce any change to the CD spectrum of the drug. However, it is possible that the low drug concentration precluded the detection of the copper–irinotecan interaction in contrast to the high irinotecan concentrations inside the liposomes upon encapsulation

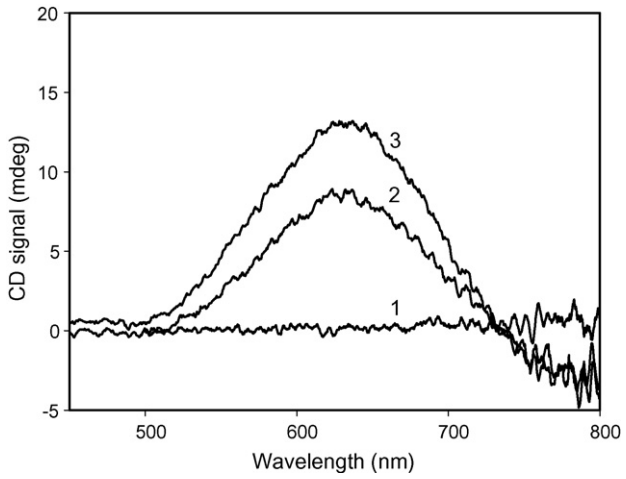

Fig. 1. Circular dichroism spectra of a solution of 2.5 mM irinotecan in water, 2.5 mM copper gluconate/4.5 mM TEA and 2.5 mM irinotecan + 2.5 mM copper gluconate/4.5 mM TEA at pH 7.0; irinotecan alone (1), copper gluconate/TEA alone (2) and irinotecan + copper gluconate/TEA (3). Spectra were recorded between 400 nm and 800 nm.

(>50 mM). This is supported by the fact that at neutral pH, concentrated solutions of irinotecan:copper gluconate/TEA at molar ratios higher than 1:1 caused the formation of a blue precipitate. Analysis of the precipitate by atomic absorption and UV-based assay revealed that the stoichiometry of irinotecan:copper in the precipitate was 1:5. The formation of a precipitate provides further evidence of a potential interaction between copper gluconate/TEA and irinotecan.

3.2. FT-IR spectroscopy

FT-IR spectroscopy was used to identify the functional groups involved in the interaction between irinotecan and copper in solution. Fig. 3 shows the FT-IR spectra of irinotecan at pH 7.0 and pH 8.7. Since irinotecan has several possible binding sites, tentative assignment of the bands of the spectra to its functional groups was first performed. The C=O stretching absorption bands appear in the region of 1870–1540 cm^{-1} (Silverstein et al., 1991). The position of the carbonyl bands is affected by several factors including intermolecular and intramolecular hydrogen bonding (Blume et al., 1988; Silverstein et al., 1991). The band at 1746 cm^{-1} is attributable to the C=O stretching vibration of the carbonyl group of the lactone ring (see Fig. 2, ring E) since it

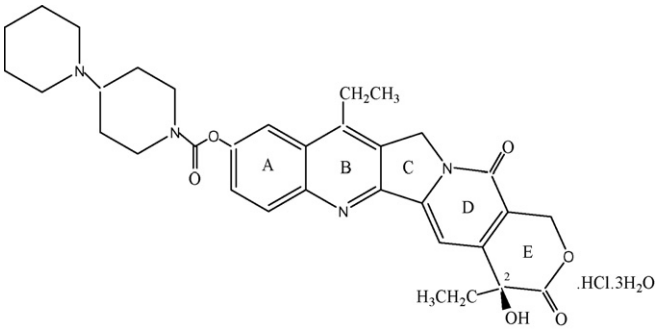

Fig. 2. Structure of irinotecan in its lactone form.

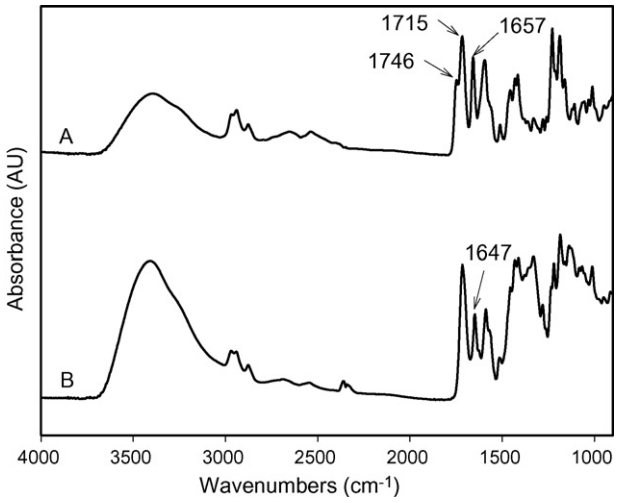

Fig. 3. FT-IR spectra of dry films of irinotecan from a solution in water. (A) Lactone form at pH 7.0 and (B) carboxylate form at pH 8.7.

is absent in the spectrum of irinotecan at pH 8.7 where the drug exists primarily in the carboxylate form. This conversion to the carboxylate form was confirmed by HPLC analysis (data not shown). Under our experimental conditions at pH 7.0, irinotecan was found to be predominantly in its lactone form. The band at 1715 cm^{-1} is assignable to the carbonyl group attached to the quinoline moiety (see Figs. 2 and 3) and was not affected by the hydrolysis of the lactone. When the drug is in its carboxylate form, the carbonyl group of ring D (see Figs. 2 and 3) is involved in hydrogen bonding interactions with the neighboring hydroxyl group, formed upon opening of the ring. This hydrogen bond caused a shift of the band at 1657 cm^{-1} to lower frequencies, which appears at 1647 cm^{-1} on the spectrum of irinotecan at pH 8.7. Thus, the band at 1657 cm^{-1} on the spectrum of irinotecan at pH 7.0 was assigned to the carbonyl group of the pyridone moiety (Fig. 2, ring D). At neutral pH, addition of copper gluconate/TEA to irinotecan at a 1:1 molar ratio did not affect the three carbonyl groups of the drug. This indicates that the interaction between irinotecan and copper gluconate/TEA likely occurred through other groups on the molecule.

We performed the sum of the spectra of copper gluconate/TEA and irinotecan and compared the resulting spectrum to that of the mixture of irinotecan and copper gluconate/TEA at the same relative concentrations. A lack of interaction between the two compounds would result in similar spectra with bands appearing at the same frequency. Fig. 4A shows that when 11 mM copper gluconate/20 mM TEA is added to 11 mM irinotecan, the band due to the hydroxyl stretching vibration at 3363 cm^{-1} is split and shifted to lower frequencies (3340–3314 cm^{-1}). The two components indicate the presence of two populations of hydroxyl groups. Comparison of this spectrum to that of irinotecan/TEA (not shown) revealed that the band at 3314 cm^{-1} and the sharp peak at 3160 cm^{-1} are due to TEA. The band at 3340 cm^{-1} is attributable to irinotecan hydrogen bonded with TEA. Fig. 4B compares the spectrum of irinotecan/copper gluconate/NaOH (11/11/16 mM, respectively) to that of the sum of the spectra of irinotecan and copper gluconate/NaOH. Contrary to what was observed

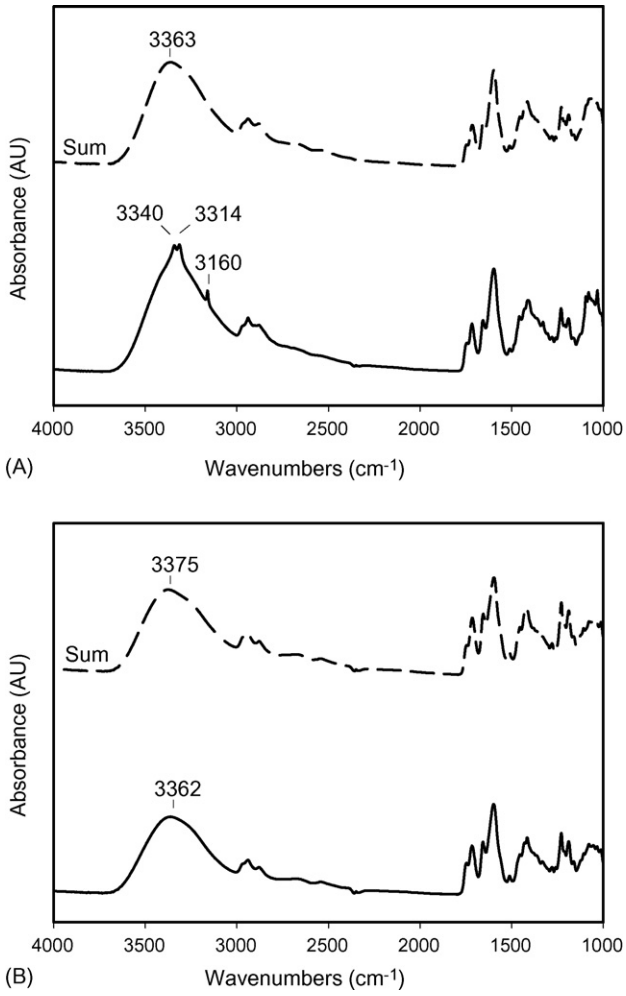

Fig. 4. FT-IR spectra of dry films from solutions in water of (A) 11 mM irinotecan + 11 mM copper gluconate/20 mM TEA (solid line), sum of the spectra of 11 mM irinotecan and 11 mM copper gluconate/20 mM TEA (dashed line). (B) 11 mM irinotecan + 11 mM copper gluconate/16 mM NaOH (solid line), sum of the spectra of 11 mM irinotecan and 11 mM copper gluconate/16 mM NaOH (dashed line).

above for irinotecan/copper gluconate/TEA, no splitting of the hydroxyl band occurred, suggesting a homogenous population of hydroxyl groups. This is consistent with the absence of TEA in that sample. The hydroxyl band appeared at a slightly lower frequency in the mixture (3362 cm^{-1}) than in the single spectra (3375 cm^{-1}). This indicates a strengthening of the hydrogen bonds with the hydroxyl groups.

3.3. Analysis of irinotecan–copper gluconate/TEA interactions in the presence of liposomes

The above FT-IR and CD data indicated that in solution, irinotecan was capable of interacting with copper gluconate/TEA. However, these techniques as well as electron paramagnetic resonance (EPR) and Raman spectroscopy did not provide any evidence of direct binding of irinotecan to copper gluconate (data not shown). The concentrations of irinotecan and copper gluconate attainable in solution, limited by the solubility of the drug in water, do not approximate the conditions of the formulation where the intra-liposomal drug concentrations can exceed 50 mM. Also, the nature of the interactions could be modulated by the presence of the lipid bilayer. Therefore, we used UV/vis and fluorescence spectroscopy to further investigate the interaction between irinotecan and copper gluconate/TEA under conditions where irinotecan was encapsulated inside liposomes containing 100 mM copper gluconate/180 mM TEA, pH 7.0. It should be noted that analysis of the liposomal formulations containing drugs and copper gluconate/TEA by cryo-electron microscopy did not reveal any morphological features that were distinct from liposomes containing only copper gluconate/TEA. As shown in Fig. 5, there was no evidence of irinotecan crystallization or precipitation inside the drug loaded liposomes and also no apparent changes in the membrane structure. In both cases, the liposomes exhibited a faceted morphology with corners, edges and textured membrane surfaces, consistent with gel phase liposomes containing low amounts of cholesterol.

3.4. UV/vis and fluorescence spectroscopy

The absorption spectra of irinotecan in the presence of liposomes containing copper gluconate/TEA, pH 7.0 is shown in Fig. 6. The spectra were recorded from samples collected at different time points during the loading of irinotecan into the liposomes at 50 °C. They are similar to the spectra of irinotecan in free solution and are characterized by four bands appearing at approximately 220 nm, 255 nm, 358 nm and 370 nm, consistent with spectra previously reported in the literature (Chourpa et al., 1998; Nabiev et al., 1998). Only the region between 280 nm and 440 nm is shown in Fig. 6 since changes in the spectra below this region were negligible. The absorbance spectra were not corrected for background scattering due to the low absorbance of drug-free liposomes in this wavelength range. When the drug was incubated with liposomes containing copper gluconate/TEA, drug encapsulation occurred. The UV/vis spectra showed that the bands at 358 nm and 370 nm shifted to 360 nm and 378 nm, respectively, and were accompanied by a decrease in intensity of the absorption band at 370 nm of irinotecan by approximately 25% (Fig. 6).

The fluorescence of irinotecan was also monitored at various time points during the irinotecan loading process. When irinotecan was added to liposomes containing 100 mM copper gluconate/180 mM TEA, pH 7.0, a 60% decrease in the fluorescence intensity at 440 nm occurred within 1 h without any apparent shift of the peak wavelength (Fig. 7A). It should be noted that the fluorescence intensity of irinotecan increased by approximately 15% over 60 min when incubated with liposomes containing sucrose phosphate buffer that were not able to encapsulate irinotecan. In addition, the emission intensity of irinotecan in sucrose phosphate buffer (no liposomes) at 50 °C decreased by approximately 8% in the first 5 min and then stabilized (data not shown).

3.5. Role of TEA in irinotecan encapsulation

Our data indicated that drug loading was negligible when NaOH was used to raise the pH of copper gluconate to 7.0.

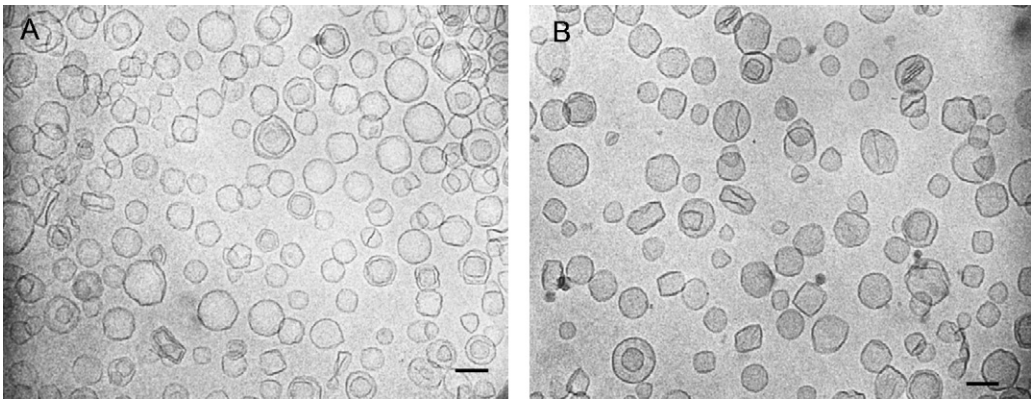

Fig. 5. Cryo-electron micrographs of liposomes containing (A) copper gluconate/TEA and (B) drugs + copper gluconate/TEA. The scale bar represents 100 nm.

Thus, we monitored irinotecan fluorescence in the presence of liposomes containing copper gluconate/NaOH following the loading method described above. The results indicated that in the presence of 100 mM copper gluconate/149 mM NaOH, the fluorescence intensity of irinotecan increased by 20% over 60 min at 50 °C (Fig. 7B). These small changes are similar to those observed above for sucrose phosphate buffer containing liposomes incubated with irinotecan and are in contrast to the quenching that occurred in the liposomes containing copper gluconate/TEA.

The fluorescence intensity of irinotecan was also monitored in the presence of liposomes containing TEA/phosphate buffer (150 mM TEA/95 mM phosphate, pH 7.0). The emission intensity of irinotecan added to the liposomes at a 0.2:1 drug to lipid ratio (mol:mol) decreased by 25% within 5 min then gradually increased to near the original fluorescence intensity within 60 min at 50 °C (Fig. 8A). Interestingly, drug encapsulation occurred and stabilized at approximately 70% efficiency, similar to that was observed above with copper gluconate/TEA containing liposomes. However, irinotecan release from TEA/phosphate containing liposomes was faster than observed with copper gluconate/TEA containing liposomes. Specifically, the half-life for irinotecan release from liposomes in plasma after i.v. injection was 8.6 h for the

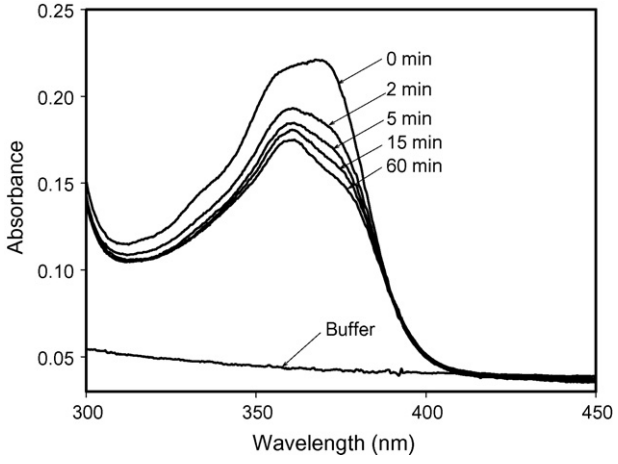

Fig. 6. Absorption spectra of irinotecan in the presence of liposomes containing 100 mM copper gluconate/180 mM TEA, pH 7.0 inside and 300 mM sucrose/40 mM phosphate, pH 7.0 outside the liposomes. Samples were collected during the loading of the drug in the liposomes at 50 °C and quenched on ice. Aliquots taken at the following time points: 0 min, 2 min, 5 min, 15 min and 60 min.

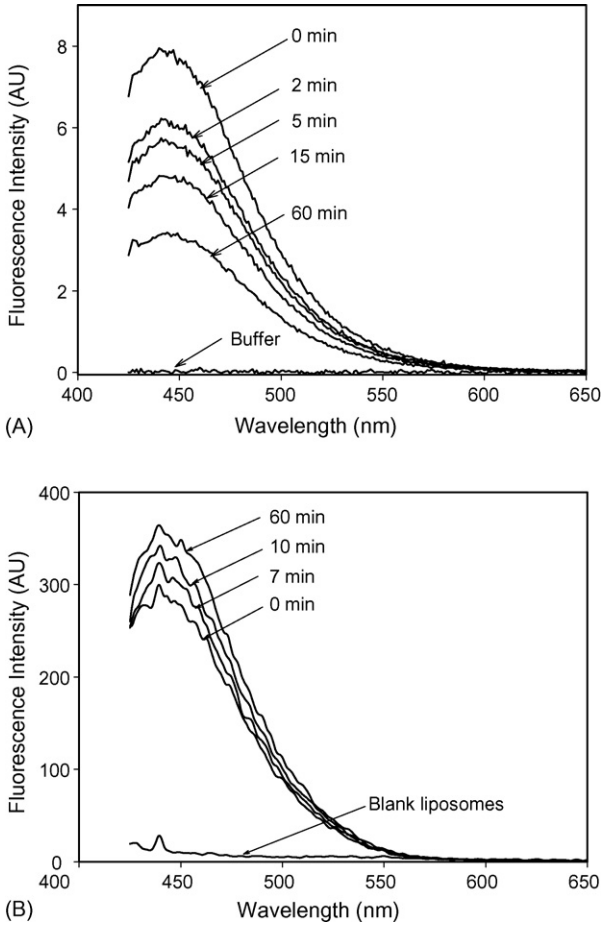

Fig. 7. Fluorescence emission spectra of irinotecan in the liposomes during loading with (A) 100 mM copper gluconate/180 mM TEA, pH 7.0 inside and 300 mM sucrose/40 mM phosphate, pH 7.0 outside the liposomes at the following time points: 0 min, 2 min, 5 min, 15 min and 60 min; (B) with 100 mM copper gluconate/149 mM NaOH, pH 7.0 inside and 300 mM sucrose/40 mM phosphate, pH 7.0 outside the liposomes at the following time points: 0 min, 7 min, 10 min and 60 min. The excitation wavelength was 400 nm. Emission spectra were collected between 425 nm and 650 nm. Spectra were recorded at room temperature.

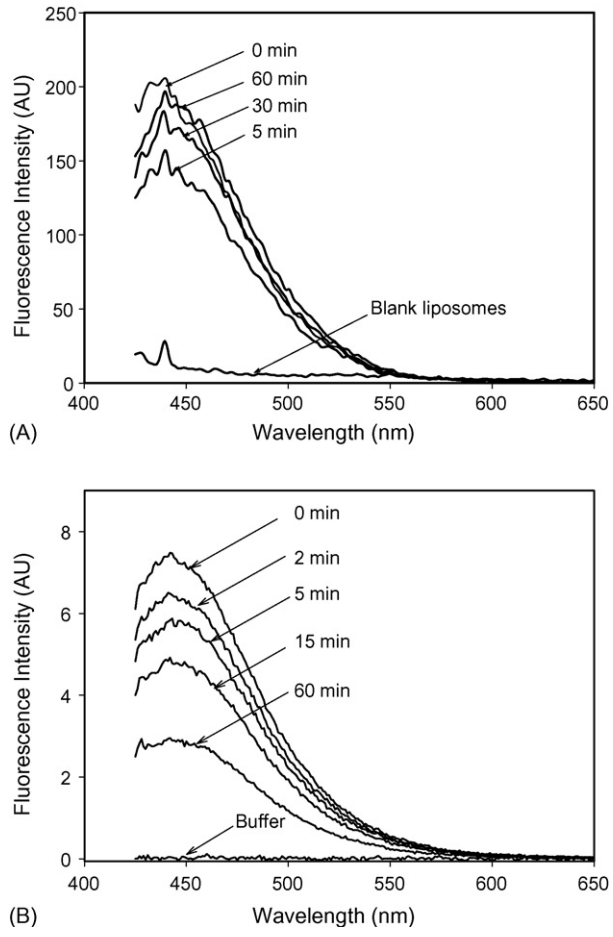

Fig. 8. Emission spectra of irinotecan during its loading into the liposomes containing: (A) 150 mM TEA/95 mM phosphate, pH 7.0 inside and 300 mM sucrose/40 mM phosphate, pH 7.0 outside at the following timepoints: 0 min, 5 min, 30 min and 60 min; (B) 10 mM sodium gluconate/180 mM TEA, pH 7.0 inside and 300 mM sucrose/40 mM phosphate, pH 7.0 outside the liposomes at the following time points: 0 min, 2 min, 5 min, 15 min and 60 min The excitation wavelength was 400 nm. Emission spectra were collected between 425 nm and 650 nm. Spectra were recorded at room temperature.

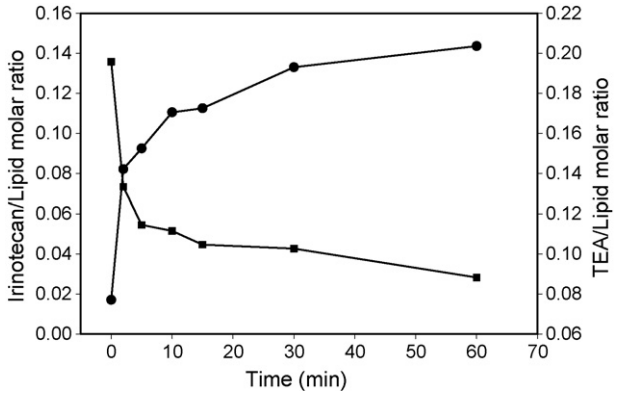

Fig. 9. Kinetic and stoichiometry correlation of TEA release (■) with irinotecan uptake (●) for liposomes containing copper gluconate/TEA.

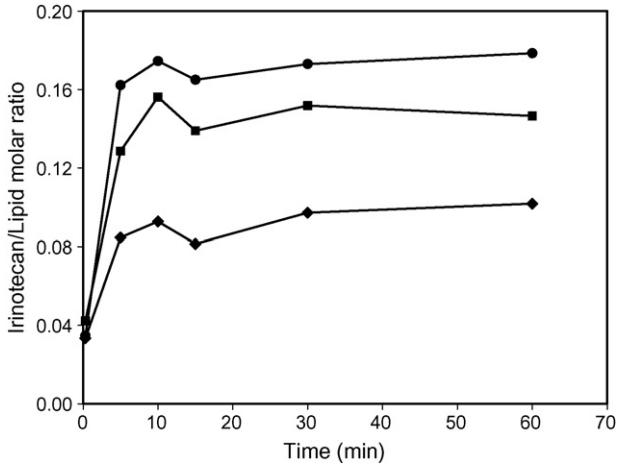

Fig. 10. Irinotecan/lipid molar ratios into liposomes containing 300 mM sucrose/40 mM phosphate, pH 7.0 outside and the following internal buffers at pH 7.0: (♦) 100 mM copper gluconate/90 mM TEA, (■) 100 mM copper gluconate/180 mM TEA and (●) 100 mM copper gluconate/270 mM TEA.

copper gluconate/TEA formulation and approximately 1 h for TEA/phosphate liposomes (data not shown). Also, room temperature dialysis of the TEA/phosphate encapsulated irinotecan resulted in drug release whereas copper gluconate/TEA liposomes exhibited no drug release over 24 h. The role of copper in inducing drug fluorescence quenching was assessed by adding irinotecan to liposomes containing 10 mM sodium gluconate/180 mM TEA, pH 7.0. Contrary to what was observed above for liposomes containing TEA/phosphate or sucrose phosphate buffer, drug fluorescence quenching occurred (Fig. 8B).

To further investigate the role of TEA in irinotecan loading, we monitored the liposome encapsulated TEA concentration relative to that of irinotecan during the encapsulation process over 1 h at 50 °C. For copper gluconate/TEA containing liposomes, TEA/lipid ratios decreased (reflecting release from the liposomes) by 0.06 μmol TEA/μmol lipid after 2 min and approximately 0.11 μmol TEA/μmol lipid after 1 h. In comparison, irinotecan/lipid molar ratios increased by 0.06 μmol irinotecan/μmol lipid and 0.13 μmol irinotecan/μmol lipid after 2 min and 60 min, respectively (Fig. 9). This observation established a kinetic and stoichiometric relationship between irinotecan encapsulation and TEA efflux. This was further supported by the fact that the amount of irinotecan encapsulated could be controlled by the amount of TEA inside the liposomes. Fig. 10 demonstrates that decreasing the concentration of TEA to 90 mM reduced the amount of drug loading by 50% while approximately 90% irinotecan encapsulation was obtained when the concentration of TEA was increased to 270 mM.

4. Discussion

It is well known that transition metals bind to drugs and the strength of the coordination depends on the nature of both the metal and the drug (Foye, 1961). For example, daunomycin and adriamycin have been shown to form strong water soluble complexes with copper (Greenaway and Dabrowiack, 1982; Feng et al., 2000). Our EPR data also indicated that irinotecan is bound to the copper ion in copper sulfate at pH 4 (data not shown). This concept was used as the basis to develop a method for loading irinotecan using copper salts at neutral pH. Copper gluconate

was chosen instead of copper sulfate because it was found to give superior drug encapsulation and retention (Tardi et al., in press). TEA was chosen to adjust the pH of copper gluconate to 7.0 due to its ability to stabilize copper solutions at neutral pH (Tardi et al., in press). In the present study, we characterized the nature of the interaction between copper gluconate/TEA and irinotecan in order to determine the role played by each component in drug encapsulation and retention.

The approach used in this work was first to characterize the interaction between irinotecan and copper gluconate/TEA in solution using various spectroscopic techniques. Self aggregation of irinotecan at elevated concentrations and the formation of a precipitate at a high drug:metal ratio limited the characterization of drug–metal interactions in solution. Therefore, we also studied the interaction between irinotecan and copper gluconate/TEA in the liposomes, which allowed characterization of the system at high intra-liposomal concentrations that reflected conditions used for the loading of irinotecan.

In free solution, our results indicated that at pH 7.0 the CD signal of copper gluconate/TEA increased upon addition of irinotecan. Since irinotecan does not have a CD signal in the visible wavelength range, the increase in intensity of the CD signal of copper gluconate suggested that the drug interacted with copper gluconate/TEA. The CD signal of copper gluconate has been proposed to result from the contribution of one C(S)–OH and two C(R)–OH groups (Gajda et al., 1998). A change in the number and type of hydroxyl groups coordinated to the copper ion, which could be induced by the addition of irinotecan, would cause a variation in intensity of the CD signal of copper gluconate (Gajda et al., 1998). Alternatively, since the binding of a chiral molecule to copper is expected to enhance its CD signal (Viles et al., 1999), the increase in intensity of the CD band may result from the contribution of irinotecan to the chirality of copper gluconate/TEA. This could occur either by the binding of irinotecan to the copper center or to one of its ligands such as gluconate and/or TEA. FT-IR data showed that irinotecan was involved in hydrogen bonding interactions with TEA. Taken together, the above observations did not reveal any evidence of irinotecan binding to copper but indicated that irinotecan interacted with copper gluconate/TEA. However, whether this interaction occurs directly with copper gluconate or indirectly via TEA is at this point unresolved.

When liposomes containing copper gluconate/TEA were incubated with irinotecan under conditions that promoted drug encapsulation, a quenching of irinotecan fluorescence was observed. For liposomes containing sucrose phosphate buffer, no drug encapsulation was obtained and a slight increase in the fluorescence emission intensity of irinotecan was seen. This latter change is consistent with a passive relocation of a portion of the drug in a more hydrophobic environment with a lower dielectric constant and is likely the result of irinotecan partitioning into the membrane (Burke et al., 1993). Similarly, when copper gluconate was pH adjusted with NaOH and trapped inside liposomes, no loading of irinotecan was observed and the drug emission intensity increased. Since neither loading nor quenching of the fluorescence were observed with copper gluconate/NaOH solutions, the presence of TEA appeared to be required for the loading of irinotecan. This is supported by the observation that accumulation of irinotecan inside the liposomes was shown to be kinetically as well as stoichiometrically correlated with TEA efflux.

The direct involvement of TEA in the loading process is further supported by the data showing that drug encapsulation occurred in liposomes containing 150 mM TEA/95 mM phosphate buffer, pH 7.0. This was accompanied by a transient quenching of the fluorescence of irinotecan followed by a subsequent increase in the emission intensity suggesting a relocation of the drug in the membrane (Burke et al., 1993). In contrast, fluorescence quenching occurred in liposomes containing sodium gluconate/TEA. This suggests that the gluconate moiety may also play a role in controlling drug retention. The quenching of irinotecan fluorescence intensity was consistent with self-association of irinotecan where planar stacking of the aromatic rings leads to self-quenching of the irinotecan fluorescence as has been documented previously (Nabiev et al., 1998) following accumulation inside the liposomes. Alternatively, irinotecan may complex with copper gluconate/TEA. However, under our experimental conditions, no evidence of irinotecan complexation with copper gluconate was found. Previous EPR studies suggested that copper could interact with the hydroxyl group of the lactone ring in camptothecin (Kuwahara et al., 1986; Brezova et al., 2003). However, the EPR spectrum of copper gluconate obtained here was not affected by irinotecan. This result argues against a direct irinotecan–copper interaction. On the other hand, our EPR data revealed that most of the copper in the 100 mM copper gluconate/180 mM TEA solution was not detectable. Thus, it is possible that EPR silent species were formed due to the formation of copper dimers (Escandar and Sala, 1992) or aggregates of drug–copper complexes resulting in antiferromagnetically coupled copper (Tachibana et al., 1987). This is consistent with the observation that irinotecan did induce changes in the EPR signal of copper sulfate at pH 4, where copper–irinotecan interactions were not complicated by the presence of copper binding ligands from gluconate and TEA.

The following mechanism is proposed for the encapsulation of irinotecan inside liposomes containing copper gluconate/TEA. Gluconate is tightly bound to copper ($K_a = 1.95 \times 10^{18}$) through its carboxyl and hydroxyl moieties as previously reported (Escandar and Sala, 1992; Gajda et al., 1998). Upon buffering of the solution with TEA, the nitrogen and/or hydroxyl groups of TEA could bind to copper (Ucar et al., 2004). When irinotecan is added to the outside of the liposome, the drug diffuses through the phospholipid bilayer in the neutral lactone form while the neutral form of TEA permeates towards the extraliposomal medium in a manner that is kinetically and stoichiometrically correlated to irinotecan uptake. At pH 7.0, based on a pK_a of 7.8 for TEA, the ratio of uncharged to charged molecules is 1:6.3. Upon movement of the uncharged form of TEA from inside the liposome, the equilibrium of TEA will shift to reprotonate TEA in the extraliposomal medium and deprotonate TEA in the liposome interior. Likewise, as irinotecan has a pKa of 8.1, it also has a significant population of both charged and uncharged molecules at pH 7.0. The ratio of uncharged to charged molecules of irinotecan at pH 7.0 is

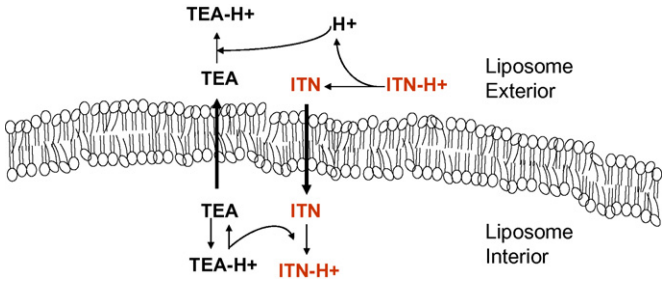

Fig. 11. Schematic of proposed neutral antiport exchange mechanism of irinotecan (ITN)/triethanolamine (TEA).

1:12.6 and the same phenomenon of transbilayer movement of uncharged molecules followed by protonation and deprotonation may be expected to occur, but in the opposite orientation relative to TEA. This creates a mutually self-buffered system where both TEA and irinotecan can readily convert between protonated and deprotonated forms to similar extents, thereby allowing active transbilayer transport without generating unfavorable electrochemical gradients that would impede further transmembrane flux of either TEA or irinotecan. A schematic representation of the proposed irinotecan/TEA neutral antiport exchange mechanism is shown in Fig. 11.

Regardless of the liposomal location of the drug complex, it appears that irinotecan interacts with neighboring drug molecules resulting in larger supramolecular complexes, which could result in the fluorescence quenching of irinotecan after encapsulation. A previous study (Aiyama et al., 1992) has correlated irinotecan self-association with alterations in absorbance spectra similar to that documented here. Such copper gluconate/TEA induced aggregates of the drug could stabilize irinotecan in its lactone form (Nabiev et al., 1998) which would account for the high lactone content inside the copper gluconate/TEA containing liposomes at pH 7.0 where significant carboxylate content would otherwise be expected. These supramolecular complexes could also account for the differences observed in irinotecan retention in copper gluconate/TEA liposomes compared to TEA/phosphate formulations. Copper gluconate is believed to play a role in modulating the flux of irinotecan and TEA across the liposomal bilayer and also appears to be important in controlling and coordinating the release of the two antineoplastic drugs floxuridine and irinotecan *in vivo* (Batist et al., 2006; Tardi et al., in press). The detailed structure of this complex, the molecular state of copper gluconate/TEA inside the liposomes and the roles played by each component in drug retention warrants further investigation and is the focus of current studies.

5. Conclusion

We utilized a variety of biophysical techniques to elucidate the potential interactions between irinotecan and copper gluconate/TEA in solution as well as in the liposomal formulation. This study helped identify the role that specific components play in irinotecan encapsulation. TEA was found to be selectively released from the liposomes in a manner that was kinetically and stoichiometrically linked to irinotecan uptake. This observation, along with others regarding conditions that affect TEA release, lead to the proposed neutral antiport exchange mechanism for the active loading of irinotecan into liposomes.

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References

Aiyama, R., Nagai, H., Sawasa, S., Yokokura, T., Itokawa, H., Nakanishi, M., 1992. Determination of self-association of irinotecan hydrochloride (CPT-11) in aqueous solution. Chem. Pharm. Bull. 40, 2810–2813.

Almgren, M., Edwards, K., Karlsson, G., 2000. Cryo transmission electron microscopy of liposomes and related structures. Colloids Surf. A: Physicochem. Eng. Aspects 174, 3–21.

Bally, M.B., Hope, M.J., Mayer, L.D., Madden, T.D., Cullis, P.R., 1988. Novel procedures for generating and loading liposomal systems. In: Gregoriadis, G. (Ed.), Liposomes as Drug Carriers. John Wiley & Sons Ltd., pp. 841–853.

Barenholz, Y., Amselem, S., Goren, D., Cohen, R., Gelvan, D., Samuni, A., Golden, E.B., Gabizon, A., 1993. Stability of liposomal doxorubicin formulations: problems and prospects. Med. Res. Rev. 13, 449–491.

Batist, G., Chi, K., Miller, W., Chia, S., Hasanbasic, F., Fisic, A., Mayer, L.M., Swenson, C., Janoff, A., Gelmon, K., 2006. Phase I study of CPX-1, a fixed ratio formulation of irinotecan (Iri) and floxuridine (Flox), in patients with advanced solid tumors. In: Proceedings of the 2006 ASCO Annual Meeting, (Abstract No. 2014).

Blume, A., Hubner, W., Messner, G., 1988. Fourier transform infrared spectroscopy of $^{13}C{=}O$-labeled phospholipids hydrogen bonding to carbonyl groups. Biochemistry 27, 8239–8249.

Boman, N.L., Mayer, L.D., Cullis, P.R., 1993. Optimization of the retention properties of vincristine in liposomal systems. Biochim. Biophys. Acta 1152, 253–258.

Brezova, V., Valko, M., Breza, M., Morris, H., Telser, J.m., Dvoranova, D., Kaiserova, K., Varecka, L., Mazur, M., Leibfritz, D., 2003. Role of radicals and singlet oxygen in photoactivated DNA cleavage by the anticancer drug camptothecin: an electron paramagnetic resonance study. J. Phys. Chem. B 107, 2415–2425.

Burke, T.G., Mishra, A.K., Wani, M.C., Wall, M.E., 1993. Lipid bilayer partitioning and stability of camptothecin drugs. Biochemistry 32, 5352–5364.

Cheung, B.C.L., Sun, T.H.T., Leenhouts, J.M., Cullis, P.R., 1998. Loading of doxorubicin into liposomes by forming Mn^{2+}-drug complexes. Biochim. Biophys. Acta 1414, 204–216.

Chourpa, I., Millot, J.-M., Sockalingum, G.D., Riou, J.-F., Manfait, M., 1998. Kinetics of lactone hydrolysis in antitumor drugs of camptothecin series as studied by fluorescence spectroscopy. Biochim. Biophys. Acta 1379, 353–366.

Conley, B.A., Egorin, M.J., Whitacre, M.Y., Carter, D.C., Zuhowski, E.G., Van Echo, D.A., 1993. Phase I and pharmacokinetic trial of liposome-encapsulated doxorubicin. Cancer Chemother. Pharmacol. 33, 107–112.

Cullis, P.R., Bally, M.B., Madden, T.D., Mayer, L.D., Hope, M.J., 1991. pH gradients and membrane transport in liposomal systems. Tibtech. 9, 57–61.

Cullis, P.R., Hope, M.J., Bally, M.B., Madden, T.D., Mayer, L.D., Fenske, D.B., 1997. Influence of pH gradients on the transbilayer transport of drugs, lipids, peptides and metal ions into large unilamellar vesicles. Biochim. Biophys. Acta 1331, 187–211.

Drummond, D.C., Noble, C.O., Guo, Z., Hong, K., Park, J.W., Kirpotin, D.B., 2006. Development of a highly active nanoliposomal irinotecan using a novel intraliposomal stabilization strategy. Cancer Res. 66, 2171–3277.

Escandar, G.M., Sala, L.F., 1992. Complexes of Cu(II) with D-aldonic and D-alduronic acids in aqueous solution. Can. J. Chem. 70, 2053–2057.

Feng, M., Yang, Y., He, P., Fang, Y., 2000. Spectroscopic studies of copper(II) and iron(II) complexes of adriamycin. Spectrochim. Acta A 56, 581–587.

Foye, W.O., 1961. Role of metal-binding in the biological activities of drugs. J. Pharm. Sci. 50, 93–108.

Gajda, T., Gyurcsik, B., Jakusch, T., Burger, K., Henry, B., Delpuech, J.-J., 1998. Coordination chemistry of polyhydroxy acids: role of the hydroxyl groups. Inorg. Chim. Acta 275–276, 130–140.

Gelmon, K.A., Tolcher, A., Diab, A.R., Bally, M.B., Embree, L., Hudon, N., Dedhar, C., Ayers, D., Eisen, A., Melosky, B., Burge, C., Logan, P., Mayer, L.D., 1999. Phase I study of liposomal vincristine. J. Clin. Oncol. 17, 697–705.

Greenaway, F.T., Dabrowiack, J.C., 1982. The binding of copper ions to daunomycin and adriamycin. J. Inorg. Biochem. 16, 91–107.

Grit, M., Crommelin, D.J., 1993. Chemical stability of liposomes: implications for their physical stability. Chem. Phys. Lipids 64, 3–18.

Haran, G., Cohen, R., Bar, L.K., Barenholz, Y., 1993. Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases. Biochim. Biophys. Acta 1151, 201–215.

Huang, S.K., Mayhew, E., Gilani, S., Lasic, D.D., Martin, F.J., Papahadjopoulos, D., 1992. Pharmacokinetics and therapeutics of sterically stabilized liposomes in mice bearing C-26 colon carcinoma. Cancer Res. 52, 6774–6781.

Kuwahara, J., Suzuki, T., Funakoshi, F., Sugiura, Y., 1986. Photosensitive DNA cleavage and phage inactivation by copper(II)-camptothecin. Biochemistry 25, 1216–1221.

Madden, T.D., Harrigan, P.R., Tai, L.C.L., Bally, M.B., Mayer, L.D., Redelmeier, T.E., Loughrey, H.C., Tilcock, C.P.S., Reinish, L.W., Cullis, P.R., 1990. The accumulation of drugs within large unilamellar vesicles exhibiting a proton gradient: a survey. Chem. Phys. Lipids 53, 37–46.

Mayer, L.D., Bally, M.B., Hope, M.J., Cullis, P.R., 1985. Uptake of antineoplastic agents into large unilamellar vesicles in response to a membrane potential. Biochim. Biophys. Acta 813, 294–302.

Mayer, L.D., Tai, L.C.L., Ko, D.S.C., Masin, D., Ginsberg, R.S., Cullis, P.R., Bally, M.B., 1989. Influence of vesicle size, lipid composition, and drug-to-lipid ratio on the biological activity of liposomal doxorubicin in mice. Cancer Res. 49, 5922–5930.

Mayer, L.D., Harasym, T.O., Tardi, P.G., Harasym, N.L., Shew, C.R., Johnstone, S.A., Ramsay, E.C., Bally, M.B., Janoff, A.S., 2006. Ratiometric dosing of anticancer drug combinations: controlling drug ratios after systemic administration dictates therapeutic activity in tumor-bearing mice. Mol. Cancer Ther. 5, 1854–1863.

Nabiev, I., Fleury, F., Kudelina, I., Pommier, Y., Charton, F., Riou, J.-F., Alix, A.J., Manfait, M., 1998. Spectroscopic and biochemical characterization of self-aggregates formed by antitumor drugs of the camptothecin family. Biochem. Pharmacol. 55, 1163–1174.

Noble, C.O., Krauze, M.T., Drummond, D.C., Yamashita, Y., Saito, R., Berger, M.S., Kirpotin, D.B., Bankiewicz, K.S., Park, J.W., 2006. Novel nanoliposomal CPT-11 infused by convection-enhanced delivery in intracranial tumors: pharmacology and efficacy. Cancer Res. 66, 2801–2806.

Ramsay, E., Alnajim, J., Anantha, M., Dicko, A., Harvie, P., Mayer, L.D., Bally, M.B., Tardi, P., 2004. A novel approach to prepare a liposomal irinotecan formulation that exhibit significant therapeutic activity *in vivo*. Proc. Annu. Meet. Am. Assoc. Cancer Res. 45 (abstract #639).

Silverstein, R.M., Bassler, G.C., Morrill, T.C., 1991. Spectrometric Identification of Organic Compounds, 5th ed. John Wiley & Sons, NY, pp. 91–142.

Tachibana, M., Iwaizumi, M., Tero-Kubota, S., 1987. EPR studies of copper(II) and cobalt(II) complexes of adriamycin. J. Inorg. Biochem. 30, 133–140.

Tardi, P.G., Gallagher, R.C., Johnson, S., Harasym, N., Webb, M., Bally, M.B., Mayer, L.D., in press. Coencapsulation of irinotecan and floxuridine into low cholesterol-containing liposomes that coordinate drug release *in vivo*. Biochim. Biophys. Acta.

Ucar, I., Yesilel, O.Z., Bulut, A., Icbudak, H., Olmez, H., Kazak, C., 2004. Bis(triethanolamine-n^{3}N,O,O′)copper(II) squarate. Acta Crystallogr. E60, m322–m324.

Viles, J.H., Cohen, F.E., Prusiner, S.B., Goodin, D.B., Wright, P.E., Dyson, H.J., 1999. Copper binding to the prion protein: structural implications of four identical cooperative binding sites. Proc. Natl. Acad. Sci. U.S.A. 96, 2042–2047.