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RESEARCH PAPER

The Role of the Transition Metal Copper and the Ionophore A23187 in the Development of Irinophore C™

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ABSTRACT

Purpose A liposomal irinotecan formulation referred to as Irinophore C relies on the ability of copper to complex irinotecan within the liposome. It is currently being evaluated for critical drug-loading parameters. Studies presented here were designed to determine the optimum copper concentration required for the effective encapsulation and retention of irinotecan into liposomes.

Methods Distearoylphosphatidylcholine/cholesterol liposomes were formulated using buffers containing various copper or manganese concentrations, and irinotecan loading was determined in the presence and absence of divalent metal ionophore A23187. The rate and extent of irinotecan encapsulation and the rate of irinotecan release from the liposomes were assessed. The amount of copper retained inside liposomes following irinotecan loading and the effect of copper on membrane permeability were determined.

Results Efficient (>98%) irinotecan loading was achieved using encapsulated copper concentrations of 50 mM. However, irinotecan release was copper concentration dependent, with a minimum 300 mM concentration required for optimal drug retention. The presence of copper increased liposomal membrane permeability.

Conclusion Results explain why irinotecan loading rates are enhanced in the presence of formulations prepared with copper, and we speculate that the Irinophore C formulation exhibits improved drug retention, due to generation of a complex between copper and irinotecan.

KEY WORDS copper · ionophore · irinotecan · liposomes

INTRODUCTION

The success of clinically approved liposomal formulations of anthracyclines (1,2), such as Caelyx, have highlighted the potential of liposomal nanoparticle (LNP) formulations to improve the therapeutic activity of selected anticancer drugs. However, the clinical development of preclinically promising LNP formulations has proven challenging. This is due to several reasons, including rising interest in molecularly targeted drugs designed to correct aberrant signaling pathways uniquely expressed in cancer cells as well as the fact that preclinical data obtained with optimized LNP formulations has not effectively predicted activities in patients. We are now pursuing clinical

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development of Irinophore C™, a liposomal formulation of irinotecan, and carefully considering the clinical development strategy to better ensure success in a Phase II setting. This strategy incorporates optimization of combination therapies as well as selection of the appropriate patient population to study. Numerous LNP irinotecan formulations are currently under investigation (3–10), and the success or failure of these formulations will be predicated on a comprehensive understanding of formulation parameters as well as biological activities. Irinophore C™ exhibits substantial therapeutic effects in multiple models of cancer (8). The mechanisms governing the therapeutic activity of Irinophore C™ involve i) stabilization of irinotecan into its active lactone form (7,11), ii) enhanced irinotecan delivery to sites of tumor growth (8), iii) increased plasma concentration over extended time periods of irinotecan as well as its more active metabolite SN-38 (8,11), and iv) the ability of this LNP irinotecan formulation to promote tumor vasculature normalization (12). A substantial amount of information has been collected to better understand the biological activities of Irinophore C™, and this database continues to expand, as the toxicity of the formulation has recently entered formal evaluation in preclinical safety studies. The studies reported here serve to supplement the biological knowledge base with information on how various formulation parameters influence the physical properties of Irinophore C™.

Irinophore C™ utilizes a combination of a pH gradient drug-loading methodology combined with encapsulated divalent metals capable of forming coordination complexes with irinotecan (7). It is now well established that divalent metals can, even in the absence of a pH gradient, facilitate the encapsulation of selected anticancer drugs with chemical groups capable of forming coordination complexes with transition metals trapped inside liposomal vesicles (3,6,7,13–17). When assessing the role of transmembrane pH gradients on this loading process, our research suggested that copper exhibited a distinct advantage over manganese (another metal commonly employed to facilitate encapsulation), in terms drug retention (6,10,17). Transmembrane pH gradients can be created in a number of ways: i) preparing liposomes using acidic aqueous buffers (14,18), ii) using aqueous solution of ammonium sulfate (5), or iii) preparing liposomes with aqueous solutions of monovalent or divalent metal ions in combination with an appropriate transmembrane ionophore (7,14,17). This third method was used to develop Irinophore C™ where the transmembrane ionophore calcimycin (also known as calcium ionophore or A23187) was added to liposomes with encapsulated metal solutions. The pH gradient is created when encapsulated divalent metals are transported out of the liposome in exchange for protons present in the external buffer. As indicated above, studies with various divalent metals demonstrated that copper was able to facilitate drug loading of topotecan (17) or irinotecan (7) even in the absence of a transmembrane pH gradient. However, in the presence of a pH gradient, there were surprising improvements in drug retention (7). Based on these observations, Irinophore C™ has been developed using formulation methods that rely on both the transmembrane pH and encapsulated copper. However, the exact mechanism behind the unique role of copper is not very well understood.

It can be argued that this formulation should be developed using the least amount of copper required to achieve optimal drug retention attributes. Although copper is known to be an essential element regulating various physiological processes in humans (19,20), if it is present in excess of cellular needs it has the potential to produce toxicities. These may involve free radical production and direct oxidation of lipids, protein and DNA (21,22). Excess copper has also been shown have roles in the development of neurodegenerative diseases (22). The primary objective of this study was to determine the minimum concentration of copper that can be used within the liposomes in order to efficiently encapsulate irinotecan into pre-formed liposomes while generating a formulation that exhibits optimal drug retention parameters following intravenous administration. In addition, the ability of copper to alter membrane permeability was evaluated. The results suggest that copper unexpectedly enhances membrane permeability as measured by sucrose permeability. However, a defined amount of copper must be retained within the liposomes after irinotecan loading in order to achieve improved drug retention.

MATERIALS AND METHODS

Materials

1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) was purchased from Avanti Polar Lipids (Alabaster, AL). 3H-cholesterylhexadecyl ether ([3H]-CHE), ^{14}C-sucrose and Pico-Fluor40 scintillation cocktail were purchased from PerkinElmer Life Sciences (Woodbridge, ON, Canada). Multi-use floating dialysis bags (Dispo-Dialyzer®) were purchased from Spectrum Labs (USA). All other chemicals used were analytical or HPLC grade. The divalent cationic ionophore A23187 (calcimycin), HEPES, Sephadex G-50, cholesterol (CH) and all other chemicals (reagent grade) were purchased from Sigma-Aldrich (Oakville, On, Canada).

Liposome Preparation

Large unilamellar vesicles (LUVs) were prepared by extrusion using DSPC and CH. Briefly, DSPC and CH

were weighed, dissolved in chloroform individually and then mixed such that the final mole ratio of the two lipids was 55:45. A non-exchangeable and non-metabolizable lipid marker, ^{3}H-CHE (5 μCi/100 μmol total lipid), was used to label the liposomes. This solution was then dried to a thin film with the help of a gentle stream of nitrogen gas. The residual chloroform was removed by placing the lipid film under high vacuum for at least 3 h. The dried lipid films were hydrated at 65°C by mixing with one of the following solutions: (i) 300 mM copper sulfate (Cu-300) (pH 3.5), (ii) 300 mM manganese sulfate (Mn-300) (pH 3.5), or (iii) different combinations of copper sulfate and manganese sulfate such that final metal concentration was 300 mM. Following hydration, samples were subjected to five freeze (liquid nitrogen) and thaw (65°C) cycles (23). The multilamellar vesicles (MLVs) obtained were extruded ten times through stacked polycarbonate filters (0.1 micron pore size) at 65°C using an Extruder™ (Northern Lipids, Vancouver, BC, Canada). The size of the LUVs generated using this method was determined using Phase Analysis Light Scattering (ZetaPALS, Brookhaven Instruments Corp., Holtsville, NY). The external buffer of LUVs was exchanged with sucrose (300 mMol/L), HEPES (20 mmol/L) and EDTA (15 mmol) (SHE buffer, pH 7.5) by running the sample through a Sephadex G-50 column equilibrated with the buffer. This EDTA-containing buffer is required to remove unencapsulated metals (copper and manganese) that may be adsorbed or bound to the outer liposomal membrane. Further, it should be noted that the loading reaction is less successful and more variable if this SHE buffer step is eliminated, suggesting that bound metal ions are influencing the surface properties of the liposomes and highlighting the important role of EDTA exposure to remove unencapsulated metals. Liposomal lipid concentration was determined by measuring ^{3}H-CHE using liquid scintillation counting (Packard 1900TR Liquid Scintillation Analyzer). Resultant liposomes contained the metal solution (unbuffered, pH 3.5) inside and were suspended in SHE buffer (pH 7.5).

Preparation of Ion Gradient and Irinotecan Loading

Prior to drug loading, the liposome solution was incubated at 30°C for 30 min in the presence or absence of A23187 (0.5 μg per 1 mg lipid). Irinotecan loading was carried out by incubating the required concentration of irinotecan hydrochloride solution (to achieve a target drug-to-lipid molar ratio ranging from 0.05 to 0.40, depending on the experiment) with the liposomal suspension at 50°C. Active loading of irinotecan into DSPC/Cholesterol liposomes has been previously studied (7), and it was demonstrated that optimal loading was achieved when the incubation temperature was 50°C. Further increases to 60°C had no impact on drug loading rates (7). Although some investigators equate optimal drug loading rate to the Tm of the bulk phospholipid (for DSPC this would be 55°C), this rarely helps predict optimal loading rates, which are more dependent on the chemical attributes of the drug being loaded. Previous studies have also been used to select 30°C as the optimal temperature for A23187 incorporation, but it should be noted that this is not based on a quantitative assessment of A23187 incorporation in the membrane, and the studies completed here have assumed that 100% of the added A23187 becomes associated with the liposomes. One-hundred-μL aliquots were removed at specific time intervals and placed onto 1 mL Sephadex G-50 spin columns pre-equilibrated with phosphate-buffered saline (PBS, pH 7.5). These columns were spun at 680×g for 3 min to separate liposomes from unencapsulated drug. Liposomes collected in the void volume were analyzed for irinotecan and lipid concentrations, and the assumption is that all drug in the void volume is liposome-associated. The columns are "calibrated" by placing solutions of free drug (no liposomes), which are then centrifuged. Under these conditions, no drug is detected in the void volume. Lipid concentrations were measured by scintillation counting, as above. Irinotecan concentrations were determined by spectrophotometric measurement of absorbance at 370 nm (Agilent/Hewlett Packard, model: 8453, Agilent Technologies. Mississauga, ON, Canada). Briefly, a portion of the samples collected from the spin columns was adjusted to 100 μL, followed by addition of 900 μL Triton X-100 (1%). This mixture was heated in a water bath at >90°C until the cloud point of the detergent was reached. Subsequently, the samples were cooled to room temperature, and the absorbance of the now clear solution was read and compared against a standard curve of known irinotecan concentrations.

Measurement of Copper Concentration

Concentration of copper present inside the liposomes was determined using atomic absorption spectrometry (AA) (AANALYST 600 PerkinElmer Instruments, Woodbridge, ON). This instrument is equipped with THGA furnace with an AS-800 Autosampler. A hollow cathode lamp (Cu-LUMINA.HCL) was used as a light source for copper detection. Briefly, liposomes were prepared and irinotecan added as described above. At specified time points (5, 10, 20, 30 and 60 min), 100-μL aliquots were removed and placed onto 1-mL Sephadex G-50 spin columns equilibrated with PBS. Liposomes were collected in the void volume after spinning the columns at 680×g for 3 min. The resulting samples were analyzed for lipid as described above and copper as follows. Aliquots were diluted in nitric acid to achieve a final nitric acid concentration of 0.1%. A portion of this sample was

injected into analysis chamber of the AA where they were aspirated and atomized. Absorbance was determined at 325 nm. Concentration of copper from the samples was determined against a freshly prepared standard curve.

In Vivo Plasma Elimination Studies

A single dose (40 mg/kg irinotecan) of the specified liposomal formulation was injected i.v. into 20–25 gm female Balb/c mice (Taconic, Hudson, NY). Four mice were used per time point, and blood samples were collected via cardiac puncture after the mice were killed by CO_2 asphyxiation. Blood was immediately placed into EDTA-containing microtainers (Becton Dickinson, NJ) and stored on ice until they could be centrifuged at 500 g for 15 min to separate plasma from blood cells. The concentration of liposomal lipid (^{3}H-CHE) in the plasma was determined by scintillation counting, and concentration of irinotecan was determined by HPLC. HPLC was conducted using a Waters Alliance HPLC system equipped with a Waters Model 717 plus autosampler, a Model 600E pump and controller and a Model 2474 Multi λ Fluorescence Detector (Waters, Milford, MA) set at an excitation wavelength of 360 nm and an emission wavelength of 425 nm. Samples were prepared by diluting into 100% ice-cold methanol. Ten µL of diluted sample was injected onto a Waters Symmetry Shield RP C18 cartridge column (100Å, particle size 5 µm; 250×4.6 mm I.D., Waters). A two-solvent mobile phase consisted of mobile phase A (75 mM ammonium acetate, 7.5 mM tetrabutylammoniumbromide, pH 6.4 adjusted with glacial acetic acid) and mobile phase B (100% acetonitrile), with an isocratic mixture of 78% A: 22% B. Each sample was run for 14 min at a flow rate of 1.0 mL/min. The drug-to-lipid ratio was estimated from these data. These animal studies were completed under an animal care protocol reviewed and approved by the University of British Columbia's Animal Care Committee. The studies met current guidelines of the Canadian Council of Animal Care.

Permeability Study

Liposomes were prepared as described above with ^{14}C-labeled sucrose added to the hydration buffer. ^{14}C-labeled sucrose has been used as a marker of lipid membrane permeability (24,25). After separating un-encapsulated sucrose from the liposomes (SHE equilibrated spin columns), the liposomes were diluted in PBS to achieve a final lipid concentration of 25 µmol/mL and then incubated at 70°C. In the development of this assay it was determined that 70°C was a temperature where sucrose release from DSPC/Chol (55:45 mol%) could be measured over a 24 h time course. At temperatures closer to the Tm of the bulk phospholipid (55°C), the sucrose release rate was too slow (occurred over 7 days, results not shown). At specified time points, 100-µL aliquots were removed and placed onto 1-mL spin columns equilibrated with PBS. Liposomes were collected in the void volume after spinning the columns at 680×g for 3 min. The amount of ^{14}C-labeled sucrose retained by the liposomes was quantified by scintillation counting.

Statistical Analysis

One-way ANOVA was performed in order to compare the results of drug-loading studies and *in vitro* and *in vivo* drug-release studies against appropriate controls. Significant differences between groups were identified using Students-Newman-Keuls multiple comparison post hoc test (GraphPad Instat software, San Diego, CA, USA). Differences between the groups were considered significant if $p<0.05$.

RESULTS AND DISCUSSION

Characterization of Irinotecan Loading

Multiple batches of liposomes have been produced, and the mean size on each batch ranged from 92 to 120 nm. The SD around each individual batch was typically up to 5%. Previous studies have reported efficient encapsulation of camptothecins such as irinotecan and topotecan into preformed liposomes containing divalent metals either in the presence or absence of an initial pH gradient (7,10,17). Drug loading using encapsulated manganese required the presence of the A23187 ionophore, whereas ionophore was not required if the encapsulated metal was copper (6, 17). These results are confirmed in Fig. 1, where increases in drug-to-lipid ratio (mol/mol) are a measure of encapsulation of drug following addition to preformed liposomes prepared in 300 mM copper sulfate (Fig. 1a) or 300 mM manganese sulfate (Fig. 1b). Drug loading was done at 50°C and in the presence (filled symbols) or absence (unfilled symbols) of A23187. These data highlight two important points. First, as stated above, efficient drug loading using 300 mM manganese as the internal buffer may only be accomplished through utilization of A23187, while liposomes prepared in 300 mM copper encapsulate drug in the presence and absence of the ionophore. Second, when using the ionophore to facilitate drug loading, the loading rate is significantly faster when using copper in the internal buffer. For example, liposomes prepared using 300 mM copper sulfate were able to encapsulate >98% of the added irinotecan within the first time point (10 min), while similar levels of loading were only achieved after 60 min for the

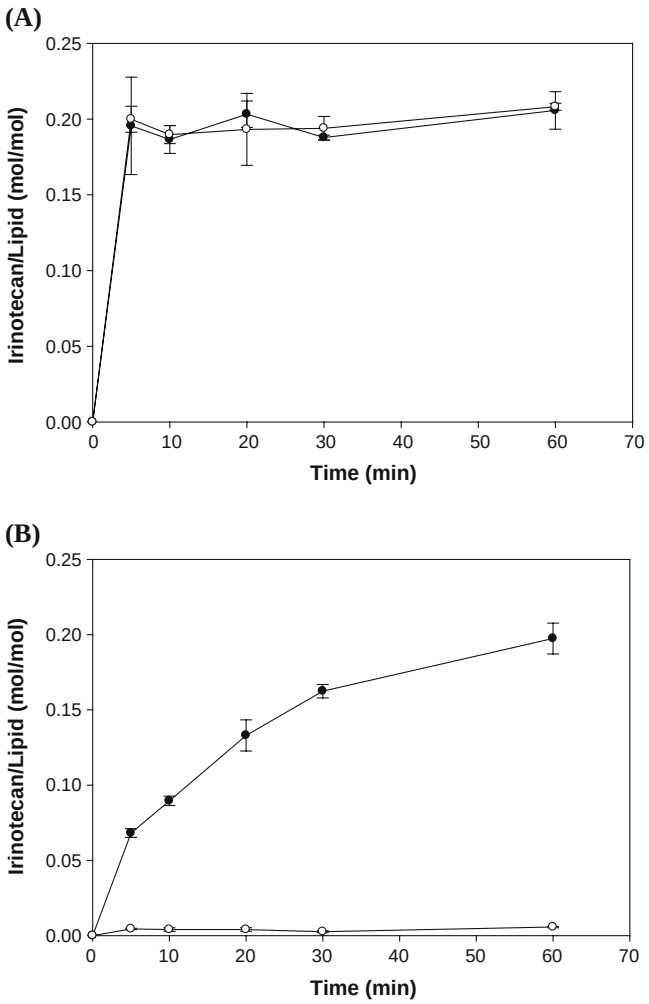

Fig. 1 $CuSO_4$ mediated irinotecan encapsulation into DSPC/CH (55:45) liposomes. (**A**) Liposomes were prepared with entrapped $CuSO_4$ (pH 3.5) plus the transmembrane ionophore A23187 (●), and $CuSO_4$ (pH 3.5) alone (○) and (**B**) Liposomes were prepared with entrapped $MnSO_4$ (pH 3.5) plus the transmembrane ionophore A23187 (●), and $MnSO_4$ (pH 3.5) alone (○). Cu^{+2} and Mn^{+2} gradients were created by exchanging the exterior liposome solution with PBS pH 7.5. Irinotecan was mixed with liposomes at a drug-to-lipid ratio of 0.2 (mol/mol) and incubated at 50°C for 60 minutes. At 5, 10, 30 and 60 minutes aliquots were fractionated on 1 mL Sephadex G-50 size exclusion column to separate encapsulated drug (collected in the eluted volume) from unencapsulated drug. Data points represent the mean ± SD ($n = 3$).

liposomes prepared in 300 mM manganese sulfate. For the copper-containing liposomes, loading rate (at 50°C) was not influenced by the presence of ionophore A23187. Irinotecan loading was also determined at 40°C, and these results have been summarized in Fig. 2. It is clear that drug loading rates are lower at 40°C compared to 50°C, but even at 40°C irinotecan encapsulation is significantly faster when copper-containing liposomes are used. We have suggested that the copper-containing formulations facilitate drug loading due to the pH gradient created following addition of A23187 as well as metal-ligand interactions and, further, that copper forms complexes with irinotecan via a coordination complex with the oxygen atoms present on the E-ring of camptothecin (17,26). This mechanism does not necessarily explain why drug loading is more rapid in the copper-containing liposomes. It is known that copper can interact with the phosphate moiety of phospholipids (16,27–29), and it may be that this copper membrane interaction is facilitating drug loading.

Influence of Entrapped Copper Concentration on Irinotecan Loading

A range of liposomal preparations prepared with varying concentrations of copper were studied to determine the optimum concentration of copper required for effective irinotecan loading. Liposomal preparations used for the initial studies were prepared using 300 mM metal sulfate solutions. This concentration was chosen to address potential issues arising from osmotic gradients that influence liposome shape and stability when mixed with solutions that are hypotonic (15). Hence, in preparations assessing the role of reduced copper concentration, the total metal concentration was maintained at or around 300 mM, where reductions in entrapped copper concentration were compensated by addition of manganese. A range of liposomal preparations with copper concentration ranging 1 mM to 300 mM were prepared, and irinotecan encapsulation efficiency was determined 10 min after drug

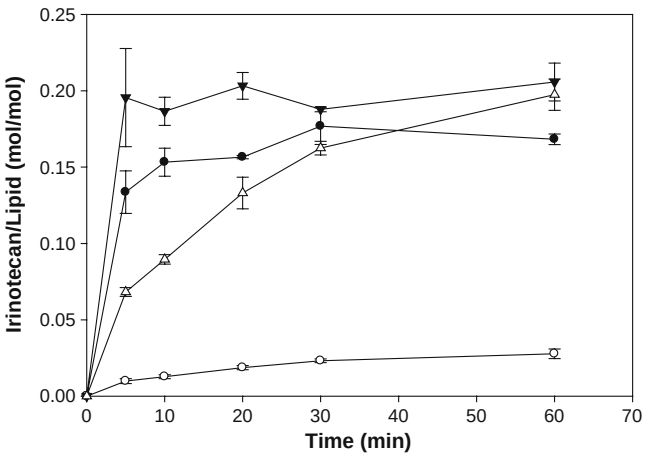

Fig. 2 $MnSO_4$ and $CuSO_4$-mediated irinotecan encapsulation into DSPC/CH (55:45) liposomes at 50°C (*triangles*) and 40°C (*circles*). Liposomes were prepared with entrapped 300 mM $MnSO_4$ (open symbols) or 300 mM $CuSO_4$ (filled symbols) (pH 3.5) plus the transmembrane ionophore A23187. Mn^{+2}/Cu^{+2} gradients were created by exchanging the exterior liposome solution with SHE pH 7.5. Irinotecan was mixed with liposomes at a drug-to-lipid ratio of 0.2 (mol/mol) and incubated at either 50°C or 40°C for 60 min. At 5, 10, 30 and 60 min, aliquots were fractionated on 1 mL Sephadex G-50 size exclusion column to separate encapsulated drug (collected in the eluted volume) from unencapsulated drug. Data points represent the mean ± SD ($n = 3$).

addition in the presence of A23187. The results are summarized in Fig. 3. As evident in Fig. 3a, reducing the encapsulated copper concentration from 300 mM to 50 mM did not have significant impact on irinotecan loading efficiency (95% *vs.* 90%). However, further reductions in encapsulated copper concentration resulted in significant decreases in drug-loading efficiency. Reducing the copper concentration from 50 mM to 25 mM reduced encapsulation efficiency from 90% to 42% ($p<0.05$), and this was further reduced as the copper concentration decreased to 10 mM. As noted in Fig. 3b, when 50 mM copper was used, the loading rate was not significantly different ($P>0.05$ at the 5, 10, and 20 min time points) from that observed when using 300 mM copper. For comparison purposes, irinotecan loading in manganese-containing liposomes was included in Fig. 3b (filled circles). The results from studies summarized in Fig. 3 were completed in the presence of A23187. To determine whether A23187 influenced loading efficiency under these conditions, irinotecan loading into liposomes prepared with 50 mM copper in the absence of A23187 was assessed. The results, summarized in Fig. 4, demonstrated that an encapsulation efficiency of only 60% can be achieved in the absence of A23187. This result highlights the importance of both the pH gradient and encapsulated copper in governing loading efficiency. It should be noted that the rate of drug loading in the absence of A23187 was essentially the same as that observed in the presence of A23187.

To further investigate the role of internal copper concentration, the amount of copper retained in liposomes following irinotecan addition was determined, and these data are reported in Fig. 5. For liposomes prepared using 300 mM copper, the amount of copper inside liposomes was reduced to 12% of the initial value within 10 min (the time point where maximum loading was noted). For the liposomal formulation prepared in 50 mM copper, the retained copper concentration was reduced to 2% of the initial (before drug addition) value. No significant changes in entrapped copper concentrations were observed after the 10-min time point (at least up to 60 min). These data demonstrate that drug loading occurs while copper is exchanged from the liposome via A23187. A23187 facilitates the exchange of copper (inside) with protons present in the external medium, and this, in turn, is important to help establish and maintain the transmembrane pH gradient.

Influence of Entrapped Copper Concentration on Drug Retention

The results thus far establish that the presence of entrapped copper facilitates the rate of irinotecan loading and that in the presence of A23187, drug-loading efficiencies for

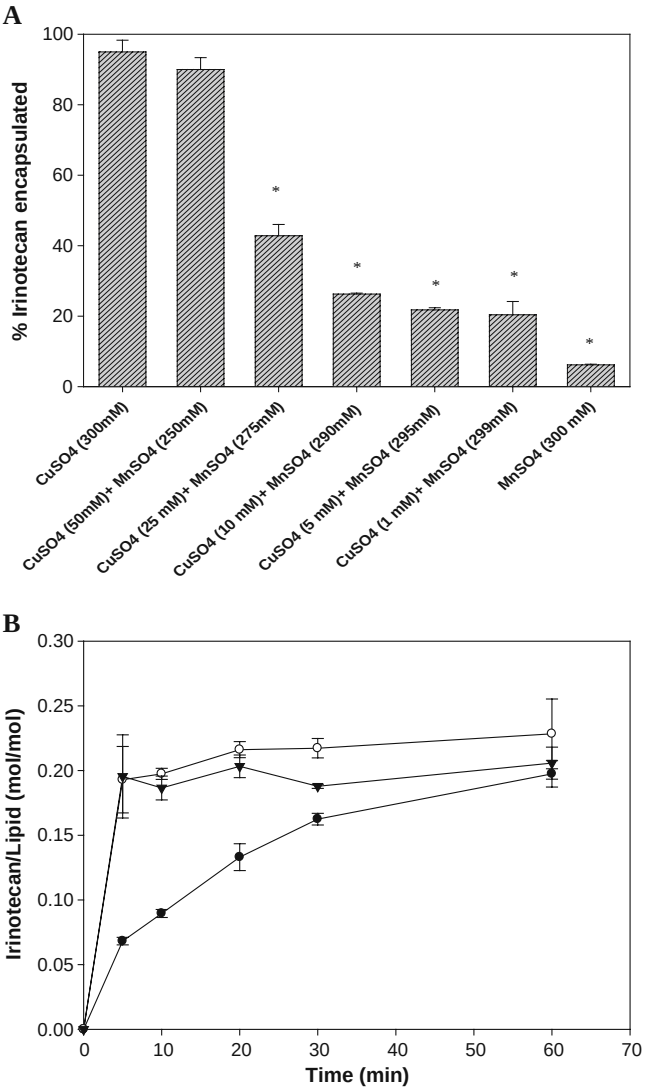

Fig. 3 Influence of entrapped copper concentration on irinotecan encapsulation into DSPC/CH (55:45) liposomes. (**A**) Liposomes were prepared using progressively reduced copper concentration. Mn^{+2} and Cu^{+2} gradients were created following addition of A23187 and subsequent exchange of the exterior liposome solution with SHE buffer, pH 7.5. Irinotecan was mixed with liposomes at a drug-to-lipid ratio of 0.2 (mol/mol) and incubated at 50°C for 10 min. After 10 min, aliquots were fractionated on 1 mL Sephadex G-50 size exclusion column prepared with PBS to separate encapsulated drug (collected in the eluted volume) from unencapsulated drug. Data points represent the mean ± SD ($n=3$). (**B**) Liposomes were prepared with *a*) entrapped $MnSO_4$ (300 mM) plus ionophore A23187 (●); *b* $MnSO_4$ (250 mM) plus $CuSO_4$ (50 mM) plus ionophore A23187 (○) or *c* $CuSO_4$ (300 mM) plus ionophore A23187 (▼). Mn^{+2} and Cu^{+2} gradients were created by exchanging the exterior liposome solution with SHE buffer, pH 7.5. Irinotecan was mixed with liposomes at a drug-to-lipid ratio of 0.2 (mol/mol) and incubated at 50°C for 60 min. At 5, 10, 20, 30 and 60 min, aliquots were fractionated on 1 mL Sephadex G-50 size exclusion column prepared in PBS to separate encapsulated drug (collected in the eluted volume) from unencapsulated drug. Data points represent the mean ± SD ($n=3$).

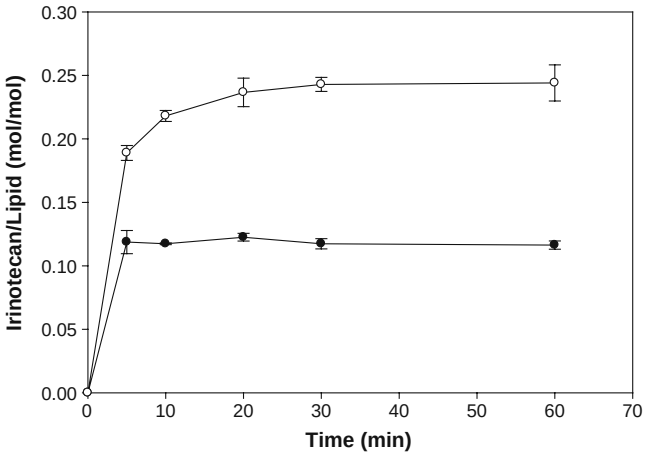

Fig. 4 Irinotecan encapsulation into DSPC/CH (55:45) liposomes. Liposomes were prepared using CuSO4 (50 mM) and MnSO4 (250 mM), and irinotecan loading was carried out *a*) in the presence of A23187 (○) and *b*) in the absence of A23187 (●). Mn^{+2} and Cu^{+2} gradients were created by exchanging the exterior liposome solution with SHE buffer, pH 7.5. Irinotecan was mixed with liposomes at a drug-to-lipid ratio of 0.2 (mol/mol) and incubated at 50°C for 60 min. At 5, 10, 20, 30 and 60 min, aliquots were fractionated on 1 mL Sephadex G-50 size exclusion column prepared in PBS to separate encapsulated drug (collected in the eluted volume) from unencapsulated drug. Data points represent the mean ± SD ($n=3$).

formulations containing 50 mM or 300 mM copper are identical. Importantly, the amount of retained copper is different in these formulations and may be as low as 1 mM when the starting formulation of copper was 50 mM and as high as 40 mM when the starting formulation was 300 mM. These data create two points of discussion. First, these data raise a potential concern for a drug-loading model emphasizing formation of a coordination complex between copper and irinotecan. Irinotecan was loaded to a drug-to-lipid ratio of 0.2 (mol:mol), and it can be estimated that the entrapped irinotecan concentration is at least 100 mM assuming the aqueous trap volume of these liposomes is 2μL/μmole lipid (30,31). Thus, when the starting formulation used contains 300 mM copper, the irinotecan-to-copper ratio after loading can be estimated to be 2.5 (mol:mol), while for the 50 mM copper formulation, this ratio would be 100 (mol:mol). This analysis again highlights the fact that both encapsulated copper and the transmembrane pH gradient influence drug loading. However, our previous studies demonstrated that drug retention was unexpectedly better in formulations prepared with copper; thus, differences in these formulations (the formulation prepared with 50 mM copper *vs.* the one prepared with 300 mM copper) could be observed if drug retention was measured.

Drug release from liposomal irinotecan formulations prepared using 300 mM and 50 mM copper was determined *in vivo* because previous work from our laboratory has demonstrated that *in vitro* release rates do not reflect those observed *in vivo*. In these studies, mice were injected via the tail vein with irinotecan-loaded liposomes at a drug dose of 40 mg/kg. At various time points, blood was collected and processed to produce plasma, and the concentration of liposomal lipid and irinotecan in the plasma was determined as described in the "Materials and Methods" section. These data were then used to calculate an irinotecan-to-lipid ratio, which was subsequently used to estimate drug release rates based on the assumption that 100% of the measured drug was associated with the liposomes in the plasma compartment. The results from this study have been summarized in Fig. 6. Four liposomal irinotecan formulations were evaluated in these studies, including ones prepared with liposomes that contained i) 300 mM copper sulfate with A23187 (filled triangle), ii) 50 mM copper sulfate plus 250 mM manganese sulfate with A23187 (open circle), iii) 300 mM manganese sulfate in presence of A23187 (filled circle), and iv) 300 mM copper in the absence of A23187 (open triangle). Consistent with the previous results (7), the plasma elimination rate of the different liposomal formulations was not significantly affected by the loading conditions used (data not shown). Thus, the differences in drug-to-lipid ratios noted in Fig. 6 are primarily a consequence of changes in the plasma elimination rate of irinotecan that had been released from the liposomal carriers. Optimal drug retention and irinotecan circulation half-life ($t_{1/2}=9.5$ hr) were observed when the liposomal irinotecan formulation was prepared with liposomes containing 300 mM copper sulfate and A23187. When the formulation was prepared with 50 mM copper

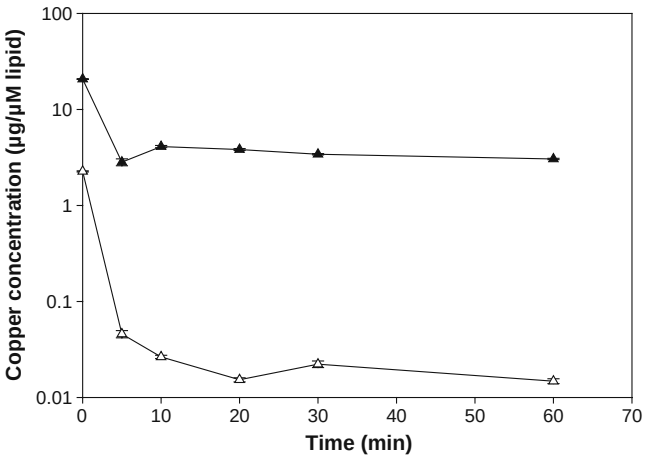

Fig. 5 Release of copper from liposomes during irinotecan loading. Liposomes were prepared using *a*) $CuSO_4$ (300 mM) (-▲-) and *b*) $CuSO_4$ (50 mM) + MnSO4 (250 mM) (-Δ-), incubated initially with ionophore A23187 at 30°C for 30 min and then with irinotecan at 50°C for 60 min. Aliquots were withdrawn at time intervals of 5, 10, 20, 30, 60 min and passed through spin columns prepared in PBS (pH 7.5) and analyzed for copper concentration. Data represent mean ± SD of triplicate measurements; if the error bars are not readily seen, then the error range is within the size of the data point used.

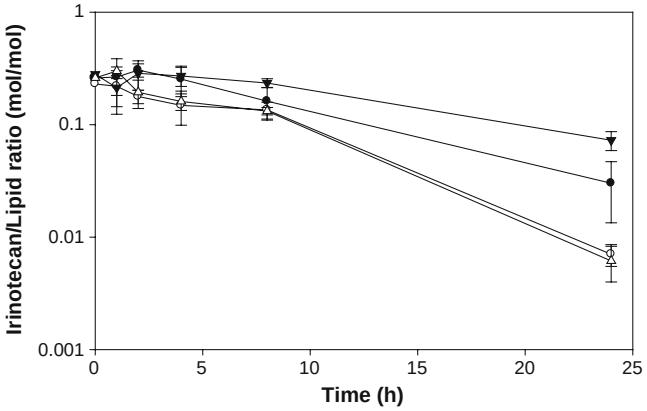

Fig. 6 Plasma elimination profiles of different liposomal Irinotecan formulations: Female Balb/C mice were injected intravenously with a single dose (40 mg/kg irinotecan) of DSPC/CH (55:45 mol%) liposomal Irinotecan formulations: 300 mM $CuSO_4$ + A23187 (▼);300 mM $MnSO_4$ + A23187 (●); 300 mM $CuSO_4$ alone (Δ); 50 mM$CuSO_4$ + 250 mM unbuffered $MnSO_4$ + A23187 (○). At the indicated time points, plasma samples were obtained (see "Materials and Methods"), and the concentration of liposomal lipid and irinotecan was determined as described in "Materials and Methods". These data were then used to calculate the irinotecan-to-lipid ratio (mol:mol) in the plasma as a function of time. Data points represent the mean ± SD ($n=4$).

sulfate and 250 mM manganese sulfate and A23187, the changes in drug-to-lipid ratio observed over time ($t_{1/2}$= 3.7 hr) were comparable to that seen following injection of liposomes prepared with 300 mM copper sulfate in the absence of A23187 ($t_{1/2}$=3.6 hr). Manganese-containing liposomes released irinotecan more rapidly ($t_{1/2}$=6.5 hr) than copper-containing liposomes.

In summary, the results indicated that 50 mM copper is sufficient to affect irinotecan loading in the DSPC/CH liposomes, but these formulations release irinotecan more rapidly *in vivo* than formulations prepared with 300 mM copper. If copper is influencing drug release rates, then it is likely that the amount of copper retained by the 50 mM copper-containing liposomes following irinotecan loading (~1 mM) is not sufficient to optimize drug retention. Previous studies established that faster drug release from irinotecan-containing liposomal formulations results in significant reductions in therapeutic activity (11). These data suggest that the 300 mM formulation prepared with A23187 exhibits the best drug retention characteristics of the formulations tested here, but these data provide no insight into why retained copper (~40 mM) improves drug retention. As indicated already, this may be due to formation of a complex between irinotecan and copper, but the stoichiometry between encapsulated irinotecan and retained copper (2.5–1) is not entirely consistent with complex formation. Although interaction of copper with camptothecins has been previously documented (26,32), a recent report completed using copper gluconate demonstrated no direct interaction between copper and irinotecan (3), although it should be noted that this formulation maintains an internal pH that is >6.5. As noted above, it has been reported that copper can bind to phospholipids (16,29), and it could be argued that the copper-lipid interaction may affect membrane permeability in a more generic way. For this reason, we assessed the influence that copper has on membrane permeability as determined using sucrose as a membrane permeable marker.

Effect of Copper on Membrane Permeability

The results in Figs. 1 and 2 clearly suggested that the presence of copper enhances irinotecan-loading rates. Faster drug-loading rates are typically associated with more rapid drug-release rates, but this is not the case for the liposomal irinotecan formulations described here. Radioactive (^{14}C labeled) sucrose was used to provide a measure of membrane permeability to help address this point. The amount of labeled sucrose retained by liposomes following incubation at 70°C was determined at specified time points. The temperature used in these studies was selected based on preliminary work establishing that sucrose release for DSPC/CH liposomes (55:45 molar ratio) could be measured within a 24 hr time course when the incubation temperature was 70°C. This temperature was well above the transition temperature of DSPC (33,34) and was expected to enhance sucrose permeability. The results, summarized in Fig. 7, indicated that for liposomes prepared in saline, encapsulated [^{14}C]-sucrose was lost gradually from the liposomes over time, with less than 20% of encapsulated sucrose being retained after the 24 hr incubation period. Somewhat surprisingly, when the liposomes were prepared with 300 mM copper, sucrose permeability was increased

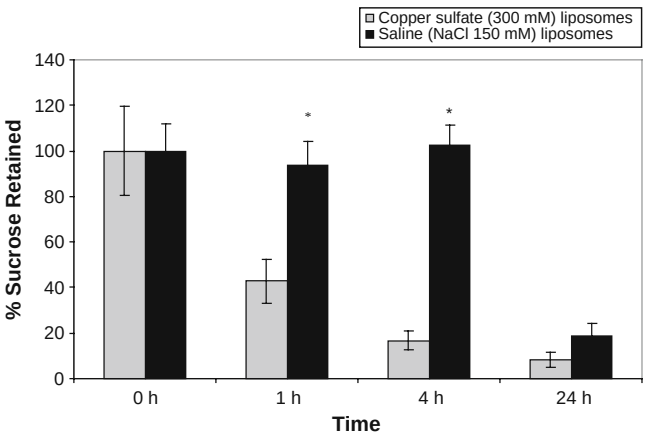

Fig. 7 Membrane permeability study of DSPC/CH liposomes: Liposomes prepared using either copper sulfate (300 mM) or saline (NaCl 150 mM) were kept in 70°C water bath. Amount of ^{14}C-labeled sucrose retained inside the liposomes was measured by passing the aliquots through a sephadex G-50 spin column equilibrated with PBS. Data points represent the mean ± SD ($n=3$).

dramatically. Liposomes prepared using saline retained greater than 90% of the initially encapsulated sucrose for at least 4 h, while liposomes prepared using 300 mM copper retained less than 20% of initially encapsulated sucrose after 4 h. These data clearly suggest that the presence of copper significantly enhanced membrane permeability. The sucrose data and the drug-loading rate data (see Fig. 1) would suggest to those skilled in the field that irinotecan release rates should be enhanced, not decreased, when copper is used. Thus, the sucrose release data is consistent with the influence of copper on irinotecan drug-loading rates, but does not explain why the copper formulation exhibits improved drug retention. Once again, these data would suggest that formation of an irinotecan-copper complex may explain why the copper, containing irinotecan-loaded formulations exhibit improved drug retention. Additional work will be required to fully characterize these purported copper-camptothecin interactions.

CONCLUSION

A summary of the results obtained here would suggest that the use of copper as an internal aqueous solution has a distinct advantage over manganese with respect to the active loading of irinotecan into DSPC/CH liposomes. The minimum concentration of copper required for efficient encapsulation of irinotecan is 50 mM. However, optimal retention of irinotecan following intravenous administration is achieved when using formulations prepared with liposomes containing 300 mM copper prior to irinotecan addition. Studies assessing the influence of copper on membrane permeability help to explain increased drug loading rates for those formulations prepared with copper; however, it remains unclear why retained copper is important to achieve improved drug retention. It can be suggested from these studies that the formulation of liposomal irinotecan being developed for clinical use (Irinophore C™) may need defined product release specifications that include retained copper.

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REFERENCES

1. Gordon AN, Fleagle JT, Guthrie D, Parkin DE, Gore ME, Lacave AJ. Recurrent epithelial ovarian carcinoma: a randomized phase III study of pegylated liposomal doxorubicin *versus* topotecan. J Clin Oncol. 2001;19:3312–22.
2. Batist G, Ramakrishnan G, Rao CS, Chandrasekharan A, Gutheil J, Guthrie T, *et al.* Reduced cardiotoxicity and preserved antitumor efficacy of liposome-encapsulated doxorubicin and cyclophosphamide compared with conventional doxorubicin and cyclophosphamide in a randomized, multicenter trial of metastatic breast cancer. J Clin Oncol. 2001;19:1444–54.
3. Dicko A, Tardi P, Xie X, Mayer L. Role of copper gluconate/triethanolamine in irinotecan encapsulation inside the liposomes. Int J Pharm. 2007;337:219–28.
4. Emerson DL. Liposomal delivery of camptothecins. Pharm Sci Technolo Today. 2000;3:205–9.
5. Haran G, Cohen R, Bar LK, Barenholz Y. Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases. Biochim Biophys Acta. 1993;1151:201–15.
6. Messerer CL, Ramsay EC, Waterhouse D, Ng R, Simms EM, Harasym N, *et al.* Liposomal irinotecan: formulation development and therapeutic assessment in murine xenograft models of colorectal cancer. Clin Cancer Res. 2004;10:6638–49.
7. Ramsay E, Alnajim J, Anantha M, Taggar A, Thomas A, Edwards K, *et al.* Transition metal-mediated liposomal encapsulation of irinotecan (CPT-11) stabilizes the drug in the therapeutically active lactone conformation. Pharm Res. 2006;23:2799–808.
8. Ramsay EC, Anantha M, Zastre J, Meijs M, Zonderhuis J, Strutt D, *et al.* Irinophore C: a liposome formulation of irinotecan with substantially improved therapeutic efficacy against a panel of human xenograft tumors. Clin Cancer Res. 2008;14:1208–17.
9. Burke TG, Xiang Xiang T, Anderson B, Latus L. Recent advances in camptothecin drug design and delivery strategies. Totowa: Humana Press Inc.
10. Tardi PG, Gallagher RC, Johnstone S, Harasym N, Webb M, Bally MB, *et al.* Coencapsulation of irinotecan and floxuridine into low cholesterol-containing liposomes that coordinate drug release *in vivo*. Biochim Biophys Acta. 2007;1768:678–87.
11. Ramsay E, Alnajim J, Anantha M, Zastre J, Yan H, Webb M, *et al.* A novel liposomal irinotecan formulation with significant anti-tumour activity: use of the divalent cation ionophore A23187 and copper-containing liposomes to improve drug retention. Eur J Pharm Biopharm. 2008;68:607–17.
12. Baker JH, Lam J, Kyle AH, Sy J, Oliver T, Co SJ, *et al.* Irinophore C, a novel nanoformulation of irinotecan, alters tumor vascular function and enhances the distribution of 5-fluorouracil and doxorubicin. Clin Cancer Res. 2008;14:7260–71.
13. Cheung BC, Sun TH, Leenhouts JM, Cullis PR. Loading of doxorubicin into liposomes by forming Mn2+-drug complexes. Biochim Biophys Acta. 1998;1414:205–16.
14. Abraham SA, Edwards K, Karlsson G, Hudon N, Mayer LD, Bally MB. An evaluation of transmembrane ion gradient-mediated encapsulation of topotecan within liposomes. J Control Release. 2004;96:449–61.
15. Dos Santos N, Waterhouse D, Masin D, Tardi PG, Karlsson G, Edwards K, *et al.* Substantial increases in idarubicin plasma concentration by liposome encapsulation mediates improved antitumor activity. J Control Release. 2005;105:89–105.
16. Kamidate T, Hashimoto Y, Tani H, Ishida A. Uptake of transition metal ions using liposomes containing dicetylphosphate as a ligand. Anal Sci. 2002;18:273–6.
17. Taggar AS, Alnajim J, Anantha M, Thomas A, Webb M, Ramsay E, *et al.* Copper-topotecan complexation mediates drug accumulation into liposomes. J Control Release. 2006;114:78–88.
18. Cullis PR, Hope MJ, Bally MB, Madden TD, Mayer LD, Fenske DB. Influence of pH gradients on the transbilayer transport of drugs, lipids, peptides and metal ions into large unilamellar vesicles. Biochim Biophys Acta. 1997;1331:187–211.

19. Burkitt MJ. A critical overview of the chemistry of copper-dependent low density lipoprotein oxidation: roles of lipid hydroperoxides, alpha-tocopherol, thiols, and ceruloplasmin. Arch Biochem Biophys. 2001;394:117–35.
20. Davis CD. Low dietary copper increases fecal free radical production, fecal water alkaline phosphatase activity and cytotoxicity in healthy men. J Nutr. 2003;133:522–7.
21. Strausak D, Mercer JF, Dieter HH, Stremmel W, Multhaup G. Copper in disorders with neurological symptoms: Alzheimer's, Menkes, and Wilson diseases. Brain Res Bull. 2001;55:175–85.
22. Valko M, Morris H, Cronin MT. Metals, toxicity and oxidative stress. Curr Med Chem. 2005;12:1161–208.
23. Mayer LD, Hope MJ, Cullis PR. Vesicles of variable sizes produced by a rapid extrusion procedure. Biochim Biophys Acta. 1986;858:161–8.
24. Franke H, Galla HJ, Beuckmann CT. An improved low-permeability *in vitro*-model of the blood-brain barrier: transport studies on retinoids, sucrose, haloperidol, caffeine and mannitol. Brain Res. 1999;818:65–71.
25. Van Veldhoven PP, Just WW, Mannaerts GP. Permeability of the peroxisomal membrane to cofactors of beta-oxidation. Evidence for the presence of a pore-forming protein. J Biol Chem. 1987;262:4310–8.
26. Kuwahara J, Suzuki T, Funakoshi K, Sugiura Y. Photosensitive DNA cleavage and phage inactivation by copper(II)-camptothecin. Biochemistry. 1986;25:1216–21.
27. Lebedev VS, Volodina LA, Deinega E, Fedorov Iu I. Structural modifications of the surface of Escherichia coli bacteria and copper-induced permeability of plasma membrane. Biofizika. 2005;50:107–13.
28. Khomutov GB, Yakovenko SA, Soldatov ES, Khanin VV, Nedelcheva MD, Yurova TV. Interaction of copper ions with stearic acid Langmuir monolayers and formation of cluster structures in monolayers and Langmuir-Blodgett films. Membr Cell Biol. 1997;10:665–81.
29. Suwalsky M, Ungerer B, Quevedo L, Aguilar F, Sotomayor CP. Cu2+ ions interact with cell membranes. J Inorg Biochem. 1998;70:233–8.
30. Elorza B, Elorza MA, Sainz MC, Chantres JR. Analysis of the particle size distribution and internal volume of liposomal preparations. J Pharm Sci. 1993;82:1160–3.
31. Maurer N, Wong KF, Hope MJ, Cullis PR. Anomalous solubility behavior of the antibiotic ciprofloxacin encapsulated in liposomes: a 1H-NMR study. Biochim Biophys Acta. 1998;1374:9–20.
32. Kuwahara J, Suzuki T, Sugiura Y. Studies on antitumor drugs targeting DNA: photosensitive DNA cleavage of copper-camptothecin. Nucleic Acids Symp Ser 1985;201–204
33. Bruggemannand EP, Melchior DL. Alterations in the organization of phosphatidylcholine/cholesterol bilayers by tetrahydrocannabinol. J Biol Chem. 1983;258:8298–303.
34. Kim JC, Bae SK, Kim JD. Temperature-sensitivity of liposomal lipid bilayers mixed with poly(N-isopropylacrylamide-co-acrylic acid). J Biochem. 1997;121:15–9.