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Research paper

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

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Abstract

We determined whether the method used to encapsulate irinotecan into 1,2-distearoyl-*sn*-glycero-phosphocholine/cholesterol (DSPC/Chol; 55:45 mol%) liposomes influenced: (i) irinotecan release rate and (ii) therapeutic efficacy. DSPC/Chol (55:45 mol%) liposomes were prepared with: (i) unbuffered $CuSO_4$; (ii) buffered (pH 7.5) $CuSO_4$; (iii) unbuffered $MnSO_4$ and the ionophore A23187 (exchanges internal metal^{2+} with external $2H^+$ to establish and maintain a transmembrane pH gradient); and (iv) unbuffered $CuSO_4$ and ionophore A23187. All formulations exhibited >98% irinotecan encapsulation (0.2 drug-to-lipid molar ratio; 10 min incubation at 50 °C). Following a single intravenous injection (100 mg/kg irinotecan) into Balb/c mice, the unbuffered $CuSO_4$ plus A23187 formulation mediated a half-life of irinotecan release of 44.4 h; a $\geqslant$4-fold increase compared to the other liposome formulations. This surprising observation demonstrated that the $CuSO_4$ plus A23187 formulation enhanced irinotecan retention compared to the $MnSO_4$ plus A23187 formulation, indicating the importance of the divalent metal. A single dose of the $CuSO_4$ plus A23187 formulation (50 mg/kg irinotecan) mediated an 18-fold increase in median $T - C$ (the difference in days for treated and control subcutaneous human LS 180 adenocarcinoma xenografts to increase their initial volume by 400%) when compared to a comparable dose of Camptosar®. Improved irinotecan retention was associated with increased therapeutic activity.
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1. Introduction

Irinotecan (CPT-11) is a water-soluble camptothecin derivative that has demonstrated clinical activity against colorectal [1,2] and small cell lung cancers [3], as well as showing promising activity in other cancer indications [4,5]. Camptothecins promote apoptosis by stabilising the cleavable complex formed between topoisomerase I (topo I) and DNA [6]. This mechanism is dependent on the integrity of the lactone ring common to all camptothecins; however, the lactone ring undergoes a pH-dependent reversible hydrolysis that favours the carboxy derivative at physiological pH [7]. Consequently, drug delivery technologies, including liposomes, have been investigated as a means to stabilise the lactone ring. Liposome formulations of topotecan and irinotecan, the two approved water-soluble camptothecins, which trap the drug in the acidic aqueous core of the liposome, have been reported [8–22]. Further, liposomes have been used to solubilise hydrophobic

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camptothecins such as SN-38 (the active metabolite of CPT-11) [23–25] and 9-nitrocamptothecin (9-NC) [13,26]. Parental formulations of liposomal lurotecan (NX211/OSI-211) [27] and an aerosol preparation of liposomal 9-NC have been tested clinically [28,29].

Weakly basic drugs such as irinotecan can be loaded into preformed liposomes that exhibit a transmembrane pH gradient (acidic inside) [30]. This approach traps the drug in an acidic environment as well as achieving efficient (>98%) loading [9] using a method that is suitable for pharmaceutical development [31]. Transmembrane pH gradients can be created directly by preparing liposomes in a well-buffered acidic solution [32]. Alternatively, ''self-generating/self-maintaining'' systems can generate a pH gradient by the presence of a transmembrane ammonium sulfate gradient [33], or by the intravesicular entrapment of a monovalent, or divalent, metal ion coupled with an appropriately selected ionophore [34]. As an alternative to these methods, we have recently described an encapsulation method that relies on formation of a copper ion/camptothecin complex, which can facilitate encapsulation of irinotecan or topotecan in the presence or absence of a transmembrane pH gradient [15,35].

The ideal drug delivery system would retain its therapeutic payload until it reaches the target site whereupon the drug would be released. Resultantly, research has focused on strategies to engineer drug retention and controlled release [36]. This study investigated the influence of transition metal-based drug encapsulation technology on irinotecan retention by a generic liposomal formulation (DSPC/Chol (55:45 mol%)). The therapeutic activity of simple liposomal anti-cancer drug formulations is dependent on the rate of release of the encapsulated drug from the liposomes following intravenous administration [37–39]. For example, increased drug retention of the vinca alkaloid vincristine was associated with increased therapeutic effects [40]; however, if vincristine retention exceeded a certain limit, a decrease in therapeutic effect was noted [41]. Similarly, liposomal formulations of the anthraquinone mitoxantrone that achieved optimal drug delivery to sites of tumour growth (e.g., formulations that retain the drug well) exhibited less therapeutic activity when compared to formulations that released the drug more rapidly and were less efficient in mediating tumour-specific drug delivery [42,43].

The studies reported herein demonstrated that the method of irinotecan encapsulation by DSPC/Chol liposomes can have surprising effects on drug retention following in vivo administration and that better irinotecan retention was associated with significant improvements in therapeutic efficacy.

2. Materials and methods

2.1. Materials

Irinotecan hydrochloride trihydrate (Camptosar®, Pfizer Canada Inc., Kirkland, QC) was purchased from the BC Cancer Agency Pharmacy. 1,2-Distearoyl-*sn*-glycero-phosphocholine (DSPC) and cholesterol (Chol) were obtained from Avanti Polar Lipids (Alabaster, AL). ^{3}H-cholesteryl hexadecyl ether (^{3}H-CHE) and ^{14}C-CHE were purchased from Perkin-Elmer Life Sciences (Boston, MA). ^{3}H-irinotecan (^{3}H-CPT-11) was manufactured by Moravek Biochemicals and Radiochemicals (Brea, CA). A23187 (calcimycin) was sourced from Sigma (Oakville, ON, Canada). All other chemicals used were of analytical or HPLC grade. The human colorectal adenocarcinoma cell line LS 180 was purchased from the American Type Culture Collection (Manassas, VA).

2.2. Liposome preparation

DSPC/Chol (55:45 mol%) large unilamellar vesicles (LUVs) were prepared as previously described [44]. Briefly, lipids were dissolved in chloroform at the required molar ratio, labelled with the non-exchangeable, non-metabolisable lipid marker ^{3}H-CHE (or ^{14}C-CHE) (5 μCi/100 μmol total lipid) and dried to a thin film under a stream of nitrogen gas. Subsequently, the lipid was placed in a high vacuum for $\geqslant$3 h to remove any residual solvent. The lipid films were hydrated at 65 °C by mixing with the appropriate metal sulfate solution prior to five cycles of freeze-and-thaw (frozen for 5 min in liquid nitrogen then thawed for 5 min at 65 °C). The multilamellar vesicle (MLV) suspensions were then extruded 10 times through stacked polycarbonate filters of 0.08 and 0.1 μm pore size at 65 °C (Extruder™, Northern lipids, Vancouver, BC, Canada). The resultant LUVs typically possessed mean vesicular diameters in the range 110 ± 30 nm as determined using Phase Analysis Light Scattering (ZetaPALS, Brookhaven Instruments Corp., Holtsville, NY). The LUVs external buffer was exchanged, using Sephadex G-50 size exclusion chromatography, with SHE buffer pH 7.5 (300 mM sucrose, 20 mM HEPES, 15 mM EDTA) or HBS buffer pH 7.5 (20 mM HEPES, 150 mM NaCl).

2.3. Accumulation of irinotecan into preformed DSPC/Chol liposomes

Where indicated, the divalent metal ionophore A23187 (1 mg/mL (1.9 mM) solution in 100% ethanol) was pre-incubated with liposomes (0.2 nmol/μmol lipid) at 50 °C for 10 min prior to drug addition, otherwise, Camptosar® (1 mM) was incubated with preformed liposomes (5 mM) at 50 °C at a drug-to-lipid ratio of 0.2:1 (mol:mol). Drug uptake was determined at indicated time points by sampling 100 μL aliquots and separating encapsulated drug from free drug using 1 mL Sephadex G-50 spin columns equilibrated with the appropriate buffer [45]. The excluded fractions, containing the liposomes, were analysed in order to determine drug-to-lipid ratios. Lipid concentrations were measured using liquid scintillation counting (Packard 1900TR Liquid Scintillation Analyzer, Perkin-Elmer, Woodbridge, ON, Canada). Irinotecan concentration was

determined by measuring absorbance at 370 nm. Briefly, a portion of the samples collected from the spin columns was adjusted to a final volume of 100 μL with HBS. Subsequently 900 μL Triton X-100 1% was added and the samples were heated in a water bath at >90 °C until the cloud point of the detergent was observed. The samples were then cooled to room temperature and the absorbance was determined against a freshly prepared irinotecan standard curve. (Agilent/Hewlett Packard UV–Vis spectrophotometer (model 8453), Agilent Technologies, Mississauga, ON, Canada). Phase Analysis Light Scattering (ZetaPALS, Brookhaven Instruments Corp., Holtsville, NY) indicated that drug-loaded liposomes did not differ in vesicular diameter compared to ''empty'' liposome controls.

2.4. Plasma elimination screening studies

Female Balb/c mice (Taconic, Hudson, NY; 20–25 g; 3 per time point) were injected i.v. with a single dose (100 mg/kg irinotecan; 450 mg/kg total lipid) of the specified liposomal formulation and the plasma concentrations of liposomal lipid (^{14}C-CHE) and irinotecan (^{3}H-CPT-11) were determined over time using dual-labelled liquid scintillation counting. The half-life of irinotecan release from the liposome formulations following i.v. administration was estimated by fitting exponential trend lines to the data plotted as the irinotecan-to-lipid ratio versus time (see Fig. 2c) and the resultant equations (for Cu pH 7.5, $y = 0.1196e^{-0.0777x}$, $r^2 = 0.9945$; for unbuffered Cu, $y = 0.2443e^{-0.0781x}$, $r^2 = 0.9980$; for Mn/A23187, $y = 0.1623e^{-0.0531x}$, $r^2 = 0.9967$; for Cu/A23187, $y = 0.2264e^{-0.0163x}$, $r^2 = 0.9963$) were used to calculate the half-life of irinotecan release based on the time taken to reach 50% of the initial drug-to-lipid ratio (SigmaPlot version 10, Systat Software Inc., San Jose, CA). The Cu^{2+}/A23187 irinotecan formulation was selected for further screening and the irinotecan (CPT-11) plasma concentration was determined in female Balb/c mice following a single intravenous dose (equivalent to 50 mg/kg irinotecan) of Cu^{2+}/A23187 liposomal irinotecan, or Camptosar® (spiked with ^{3}H-CPT-11). The plasma levels of irinotecan (^{3}H-CPT-11) were determined at the indicated time points as described above.

2.5. Cryo-transmission electron microscopy

The cryo-TEM images were obtained by Göran Karlsson and Katarina Edwards, Department of Physical Chemistry, Uppsala University, Sweden. Briefly, radioactive-free DSPC/Chol liposomes were loaded with irinotecan using the unbuffered 300 mM $CuSO_4$ plus A23187 loading methodology at a drug-to-lipid molar ratio of 0.2 as described above. In a chamber of controlled temperature (25 °C) and humidity, 1–2 μL of liposome sample was deposited on copper grids coated with a holey cellulose acetate butyrate polymer. Excess liquid was then blotted away with filter paper. The samples were quickly vitrified by plunging into liquid ethane and transferred to liquid nitrogen keeping the sample below 108 K, therefore minimising sample perturbation and the formation of ice crystals. The grid was transferred to a Zeiss EM902 transmission electron microscope where observations were made in a zero-loss bright-field mode and an accelerating voltage = 80 kV.

2.6. Thin-layer chromatographic (TLC) analysis of the lactone and carboxy forms of liposomal irinotecan

Irinotecan controls and liposomal irinotecan prepared by the Cu^{2+} plus A23187 drug encapsulation technology were solubilised in $CHCl_3$:MeOH (1:1 v/v) and spotted on a TLC plate. The lactone and carboxy forms of the drug were separated by exposing the TLC plate initially to a mobile phase of $CHCl_3$:MeOH:acetone (9:3:1 v/v/v) for 30 min. The plate was then dried and subjected to a further 4-h exposure to a second mobile phase comprising butanol:acetic acid:water:acetone (4:2:1:1 v/v/v/v). The drug species were visualised under UV light.

2.7. Efficacy and tolerability studies

Female RAG2-M mice (129S6/SvEvTac-*Rag2*^{tm1Fwa}, Taconic, Hudson, NY; 20–25 g; 6 per group) were inoculated with 1×10^6 (50 μL) LS 180 cells subcutaneously on the back. Once the tumour was established and reached a size of ~100 mg, a single dose (50 mg/kg) of Camptosar® or the indicated liposomal irinotecan formulation was administered via the lateral tail vein (200 μL). Tumour growth was monitored daily with calipers and the measured dimensions (mm) converted to tumour weight (mg) using the equation: length × (width^2)÷ 2 [46].

All animal studies were conducted in accordance with the Canadian Council on Animal Care's Guide to the Care and Use of Experimental Animals. Mice were housed under standard conditions with enrichment and had access to food and water *ad libitum*. All animals were observed at least two times per day for morbidity, more if deemed necessary during the pre-treatment and treatment periods. Signs of ill health were based on body weight loss, change in appetite, and behavioural changes such as altered gait, lethargy and gross manifestations of stress. Animals were terminated (CO_2 asphyxiation) if signs of severe toxicity or tumour-related illness were observed and a necropsy performed to assess other signs of toxicity.

2.8. Statistical analysis

LS 180 efficacy data were analysed using SPSS 13.0 (SPSS Inc, Chicago, IL). The time taken for s.c. LS 180 tumours to increase in volume by 400% was analysed using Kaplan-Meier curves for survival analysis. This analysis allows modelling to the time event data (i.e., size increase by 400%) in the presence of censored cases (i.e., animals who never reached this end-point because of, for example, tumour ulceration). Treatment and control groups were compared using a log-rank test.

3. Results

3.1. Efficiency of metal ion-mediated irinotecan encapsulation

We have reported that irinotecan encapsulation by DSPC/Chol liposomes containing an unbuffered 300 mM $MnSO_4$ solution was dependent on use of the divalent cation ionophore A23187 [12]. More recently, we have investigated irinotecan encapsulation into DSPC/Chol liposomes mediated by entrapped 300 mM $CuSO_4$ solutions that were unbuffered or buffered to pH 7.5 [35]. In this present study, we compare these different irinotecan formulations to DSPC/Chol liposomes containing unbuffered 300 mM $CuSO_4$, where the ionophore A23187 was added prior to addition of the drug. This loading method was analogous to the one described previously for irinotecan or topotecan encapsulated into Mn^{2+}-containing liposomes [12,15]. A23187 is a divalent metal ionophore that drives drug encapsulation by generating and maintaining a transmembrane pH gradient through the electroneutral exchange of Cu^{2+} or Mn^{2+} from the liposome interior for $2H^+$ from the external solution. It was assumed that drug loading or drug release rates would not be influenced by the choice of the encapsulated divalent metal ion that was coupled with A23187 to promote irinotecan encapsulation.

Fig. 1(inset), indicates that irinotecan encapsulation was comparable ($\geqslant$98%) for all liposomal formulations tested when irinotecan (Camptosar®) was incubated (50 °C for 10 min) with the liposomes at a drug-to-lipid ratio of 0.2:1 (mol:mol). The time course data (Fig. 1) demonstrated that the rate of loading and the stability of the liposomal irinotecan formulations were also comparable over a 1-h incubation period at 50 °C.

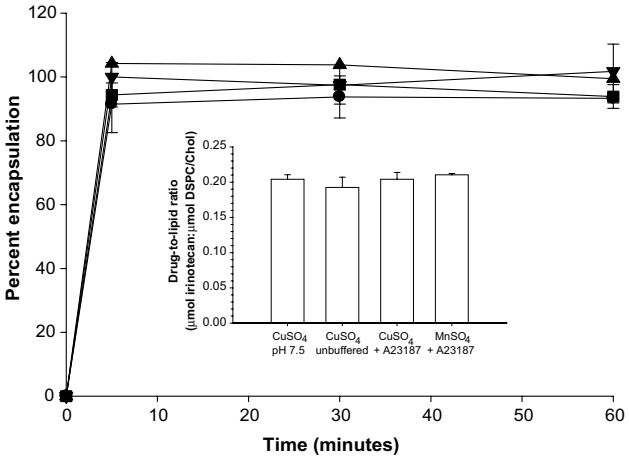

Fig. 1. The rate and extent of irinotecan encapsulation by DSPC/Chol liposomes (55:45 mol%) with entrapped copper ion and manganese ion solutions. Liposomes (5 mM) were suspended in SHE buffer (300 mM sucrose, 20 mM HEPES, 15 mM EDTA, pH 7.5) and, where indicated, were incubated with the ionophore A23187. The rate and percent of irinotecan encapsulation, at an initial drug-to-lipid ratio (mol:mol) of 0.2 and an incubation temperature of 50 °C, is shown as a function of drug-loading conditions: 300 mM $CuSO_4$, 20 mM HEPES, 220 mM TEA, pH 7.5 (●); unbuffered 300 mM $CuSO_4$ (■); unbuffered 300 mM $MnSO_4$ + A23187 (▲); unbuffered 300 mM $CuSO_4$ + A23187 (▼). (Inset) Drug-to-lipid ratios achieved after an incubation with irinotecan at an initial drug-to-lipid ratio (mol:mol) of 0.2 at 50 °C for 10 min. The encapsulation efficiency (irinotecan-to-lipid ratio (mol:mol)) was determined as described in the Section 2. Data points represent the mean ± SD of at least three independent experiments.

3.2. Retention of irinotecan by different liposome formulations in vivo

The plasma elimination profiles for the four liposomal irinotecan formulations were compared in a preliminary in vivo screen to determine whether the rate of irinotecan release from these liposomes was influenced by the drug loading method used. For economic and ethical reasons, our research team uses 3-time points when screening for differences in the elimination behaviour associated with formulation changes. The results, summarised in Fig. 2, were obtained following i.v. administration of the specified liposomal irinotecan formulation (irinotecan 100 mg/kg; total lipid 450 mg/kg) to female Balb/c mice. At the indicated time points, the concentrations of liposomal lipid (Fig. 2a) and irinotecan (Fig. 2b) in the plasma were determined as described in Section 2. The results have been presented as % injected dose in plasma, assuming a mouse plasma volume of 1 mL/22 g mouse [47]. As would be expected for liposomal formulations prepared with identical lipid composition (DSPC/Chol; 55:45 mol%), the liposome elimination rates were not substantially different suggesting that: (i) the different internal environments of the liposome formulations did not influence the rate of liposome elimination from the plasma compartment, and (ii) prior exposure to the ionophore A23187 did not influence the rate of liposome elimination from the plasma compartment (compare filled circles to filled inverted triangles). Finally, the results suggest that 35% to 40% of the injected liposomal lipid dose, regardless of the encapsulation method used, was still within the plasma compartment 24 h after i.v. administration.

In contrast, the plasma elimination rate of irinotecan was dependent on the drug loading conditions. The slowest irinotecan elimination rates were obtained when the drug was encapsulated in liposomes containing unbuffered $CuSO_4$ and added A23187 (filled inverted triangles). At the 24-h time point, 25% of the initial irinotecan dose was detectable in the plasma compartment following injection of this formulation. Surprisingly, the rate of irinotecan elimination from the plasma compartment was faster for formulations prepared using liposomes containing $MnSO_4$ and added A23187 formulation (filled triangles), where only 8% of the injected drug dose was obtained in the plasma compartment at the 24-h time point. A similar plasma release profile was observed for irinotecan formulated using unbuffered $CuSO_4$ (filled squares). In contrast, liposomes prepared with the buffered $CuSO_4$ solution

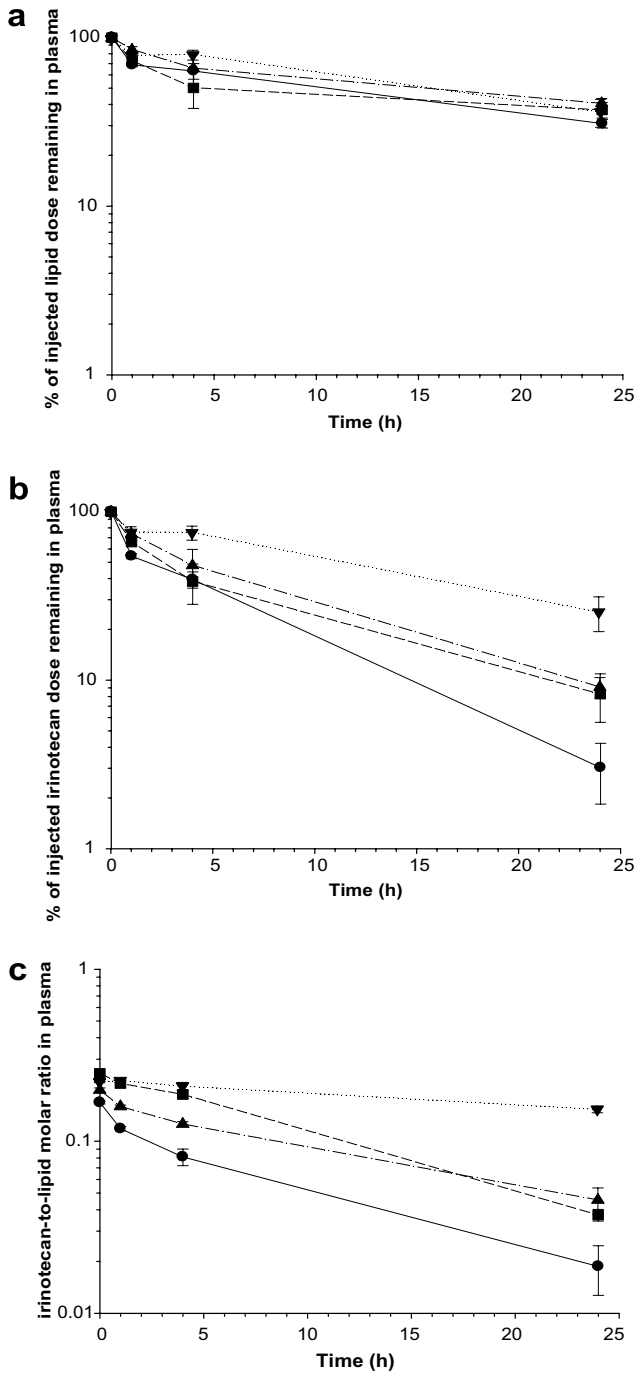

Fig. 2. Plasma elimination profiles for different liposomal irinotecan formulations. Female Balb/c mice were injected intravenously with a single dose (100 mg/kg irinotecan; 450 mg/kg lipid) of DSPC/Chol (55:45 mol%) liposomal irinotecan formulations: 300 mM $CuSO_4$, 20 mM HEPES, 220 mM TEA, pH 7.5 (●); unbuffered 300 mM $CuSO_4$ (■); unbuffered 300 mM $MnSO_4$ + A23187 (▲); unbuffered 300 mM $CuSO_4$ + A23187 (▼). (a) The percentage of the initial dose of liposomal lipid (^{14}C-CHE) in plasma as a function of time. (b) The percentage of the initial dose of irinotecan (^{3}H-CPT-11) in plasma as a function of time. (c) The irinotecan-to-lipid molar ratio in plasma as a function of time. Data points represent the mean ± SD ($n = 3$).

mediated the fastest rate of irinotecan elimination, where 3% of the injected dose was within the plasma compartment 24 h while after administration. Given comparable liposome elimination rates (Fig. 2a), the differences in plasma irinotecan concentrations reflect differences in drug retention within the liposomes following i.v. administration. This can be illustrated by using the plasma drug and liposomal lipid concentrations to calculate the drug-to-lipid ratio in the plasma as a function of time, which assumes that the vast majority of irinotecan in the plasma compartment is associated with circulating liposomes. The results are shown in Fig. 2c and clearly demonstrate that optimal drug retention is achieved when the drug is encapsulated in liposomes containing $CuSO_4$ and added A23187 (filled inverted triangles). The initial (prior to administration) drug-to-lipid ratio (mol:mol) for this formulation was 0.2 and 24 h after administration the estimated drug-to-lipid ratio (mol:mol) was 0.15, which equated to a half-life of irinotecan release from the liposomes of 44.4 h (see Section 2). In comparison the formulation prepared using liposomes containing $MnSO_4$ and added A23187 exhibited a drug-to-lipid ratio of 0.05 at 24 h post administration and an irinotecan release half-life equal to 10.0 h. These results clearly demonstrated that the drug loading method influenced irinotecan release rates from the liposomes following i.v. administration. Consequently, the Cu^{2+}/A23187 liposome formulation of irinotecan was selected for further investigation.

The plasma concentration of irinotecan following a single i.v. dose (equivalent to 50 mg/kg of irinotecan) of the Cu^{2+}/A23187 liposomes was compared to that of Camptosar® over 24 h (Fig. 3). The liposome formulation mediated a 180-fold increase in irinotecan present in the plasma 1 h after injection. At the 8 h time point the irinotecan concentration associated with the liposomes was greater than 15,000 times that recorded following Camptosar® administration. Irinotecan levels in the plasma of animals injected with Camptosar® were below detectable levels 24 h after i.v. administration.

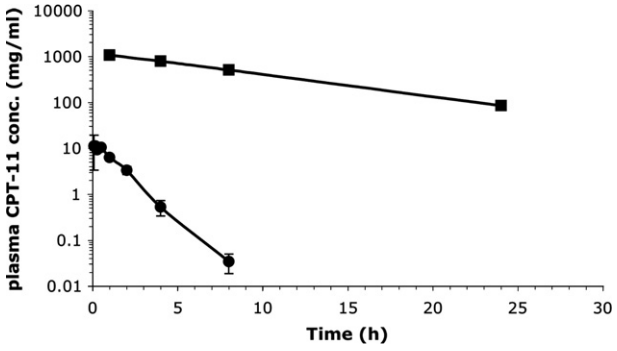

Fig. 3. Plasma irinotecan (CPT-11) concentration in mice following a single intravenous dose of Camptosar® or Cu^{2+}/A23187 liposomal irinotecan. Female Balb/c mice were injected intravenously with a single dose (equivalent to 50 mg/kg irinotecan) of Camptosar® or DSPC/Chol (55:45 mol%) Cu^{2+}/A23187 liposomal irinotecan. The plasma concentration of irinotecan (^{3}H-CPT-11) was determined as a function of time (see Section 2). Data points represent the mean CPT-11 concentration ± SD ($n = 3$).

3.3. The intravesicular morphology and conformation of irinotecan encapsulated in liposomes using the $CuSO_4$ plus A23187 loading method

Recent studies have suggested that increased liposomal retention of vinorelbine and ciprofloxacin was achieved by the formation of intravesicular drug-arylsulfonate complexes [48]. Cryo-TEM was used to determine whether greater irinotecan retention could be explained by formation of an intravesicular precipitate within DSPC/Chol liposomes prepared using the unbuffered $CuSO_4$ plus A23187 drug-loading method. The results are shown in Fig. 4, with (panel A) representative of typical liposome morphology in the absence of drug where the vesicular diameter was comparable to that estimated using Phase Analysis Light Scattering (see Section 2). When irinotecan was encapsulated using $CuSO_4$ plus A23187 (panel B), there was evidence of dark intravesicular spots (arrows). The presence of the electron-dense spots was, however, not uniformly seen in all liposomes. These observations were consistent with previous results from our laboratory showing similar intravesicular precipitates in irinotecan formulations prepared using unbuffered $CuSO_4$ alone, and $CuSO_4$ buffered to pH 7.5 [35]. This suggested that the presence of the precipitate does not explain differences in drug release rates for these formulations. The precipitates observed in the irinotecan formulations were distinct from those observed for liposomal formulations of topotecan, which under similar experimental conditions, exhibit linear "needle-like" precipitates and distinctive "coffee bean" liposome morphology [15].

Fig. 5 shows a representative TLC image of irinotecan extracted from liposomes prepared using the Cu^{2+} plus A23187 encapsulation method. The irinotecan controls are on the left-hand side of the TLC plate and show the differences in migration between the carboxy (pH 10.0) and lactone (pH 2.0) forms of the drug. Irinotecan isolated from the liposomes had a signal equivalent to the pH 2.0 control with no evidence of a migratory band comparable to the pH 10.0 control. This indicated that the vast majority of the irinotecan encapsulated using Cu^{2+}/A23187 active loading was in the active lactone form. These results are similar to obtained when unbuffered Cu^{2+} and Cu^{2+} pH 7.5 solutions were used to encapsulate irinotecan in DSPC/Chol liposomes [35].

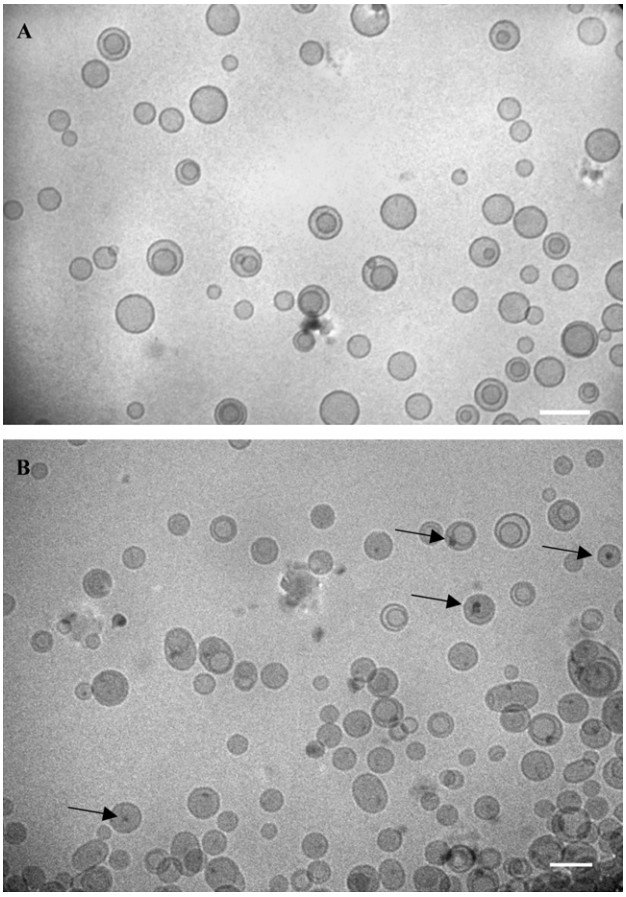

Fig. 4. Cryo-TEM images of DSPC/Chol liposomes loaded with irinotecan using the unbuffered $CuSO_4$ plus A23187 loading methodology. (A) A representative cryo-TEM image of DSPC/Chol liposomes prepared with unbuffered 300 mM $CuSO_4$ in the presence of A23187. (B) The same liposome conditions as described for (A), except that irinotecan has been encapsulated at a drug-to-lipid molar ratio of 0.2. The arrows indicate dark intravesicular spots. The bars in the photomicrographs = 200 nm.

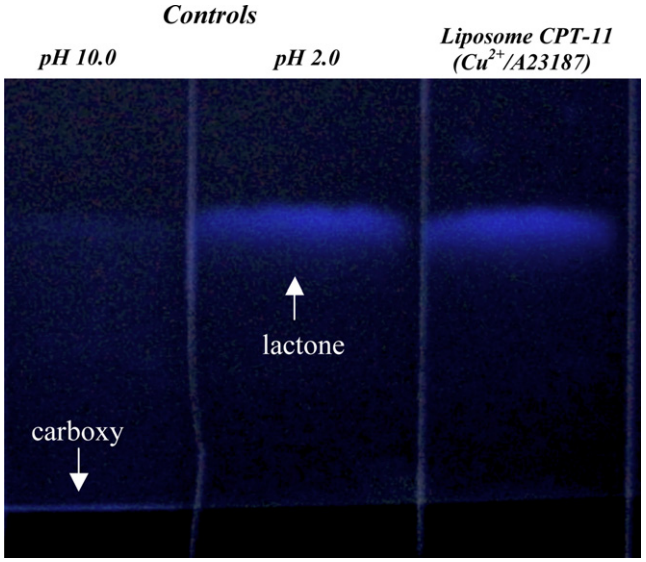

Fig. 5. Thin-layer chromatographic (TLC) analysis of irinotecan conformation following liposomal encapsulation using the Cu^{2+}/A23187 loading method. Irinotecan controls and the liposomal sample were solubilised in $CHCl_3$:MeOH (1:1 v/v) and spotted on a TLC plate. The lactone and carboxy forms of the drug were separated by exposing the TLC plate initially to a mobile phase of $CHCl_3$:MeOH:acetone (9:3:1 v/v/v) followed by a mobile phase of butanol:acetic acid:water:acetone (4:2:1:1 v/v/v/v). The drug was visualised under UV light.

3.4. The relationship between irinotecan retention and therapeutic efficacy for different copper-based liposomal irinotecan formulations

We have previously described the therapeutic effects of liposomal (DSPC/Chol; 55:45 mol%) irinotecan encapsulated in Mn^{2+}-containing liposomes with added A23187 [12], and we have demonstrated here that the rate of drug release from this liposomal formulation is identical to that obtained for liposomal (DSPC/Chol; 55:45 mol ratio) irinotecan formulations prepared using liposomes containing unbuffered $CuSO_4$ (see Fig. 2). Consequently, we focused on the three formulations prepared using Cu^{2+}-containing liposomes, which exhibited three different rates of drug release (Fig. 2c), to determine whether systemic irinotecan release rates influenced anti-tumour activity.

The therapeutic effectiveness of a single intravenous dose (50 mg/kg CPT-11) of each liposomal irinotecan formulation was compared to that of Camptosar® for the treatment of female RAG2-M mice bearing subcutaneous human LS 180 colorectal adenocarcinoma xenografts (Table 1). The time taken for each treated/control tumour to increase to 400% (400% TGI) of its initial size (~100 mg) was used as a measure of efficacy. Table 1 shows that a single dose of Camptosar® mediated a mean time to 400% TGI of 21.8 days, which was comparable to the saline control group. In contrast, the copper-based liposome formulations delayed tumour growth (time to 400% TGI) compared to saline control. The longest measured mean time to 400%TGI was 28.6 days following treatment with the liposomal irinotecan formulation prepared using the Cu^{2+} plus A23187 loading procedure (Table 1).

To investigate the relationship between the rate of irinotecan release from the different liposome formulations and anti-tumour efficacy, the values for median $T - C$ (the difference in days for treated and control tumours to increase in size 400%; Table 1) were plotted against 1/half-life of irinotecan release estimated from the curves shown in Fig. 2c (see Section 2) [41]. The results are shown in Fig. 6.

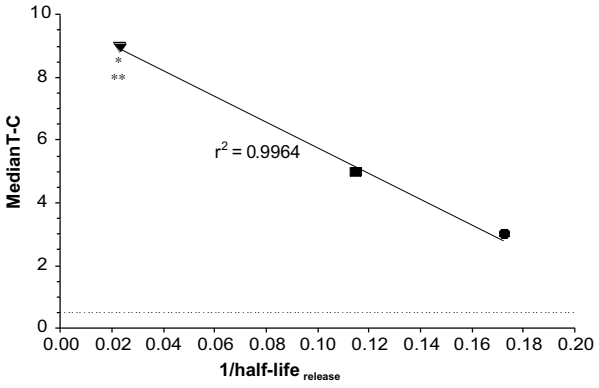

Fig. 6. The therapeutic efficacy of liposomal irinotecan correlates with drug retention. The median $T - C$ is plotted as a function of 1/half-life$_{release}$ (h^{-1}) for DSPC/Chol (55:45 mol%) liposomal irinotecan formulations: 300 mM $CuSO_4$, 20 mM HEPES, 220 mM TEA, pH 7.5 (●); unbuffered 300 mM $CuSO_4$ (■); unbuffered 300 mM $CuSO_4$ + A23187 (▼). The median $T - C$ represents the difference in time taken (days) for drug treated LS 180 tumours to increase in size by 400% of their initial size compared to saline treated controls (see Table 1). 1/half-life$_{release}$ is calculated from the half-life of irinotecan release values shown in Fig. 2c (see also Section 2). There is a positive correlation between median $T - C$ and 1/half-life$_{release}$ with an $r^2 = 0.9964$. $^{*}p < 0.05$ compared to Camptosar® and $CuSO_4$ pH 7.5. $^{**}p < 0.08$ compared to $CuSO_4$ unbuffered. The dotted line indicates the median $T - C$ associated with Camptosar®.

Analysis of these data demonstrated a positive correlation (solid line; $r^2 = 0.9964$) between 1/half-life of irinotecan release (as a measure of irinotecan retention) and median $T - C$ (representing therapeutic effectiveness) in a xenograft model of human colorectal cancer. The $CuSO_4$ plus A23187 formulation (filled triangle) mediated the slowest irinotecan release rate (1/half-life of irinotecan release = 0.02) and promoted the greatest anti-tumour activity ($T - C = 9$). The dotted line in Fig. 6 corresponds to the median $T - C$ for Camptosar®, and formulations with poor drug retention (i.e., 1/half-life$_{release}$ values greater than those plotted for the copper-based preparations) would also demonstrate reduced therapeutic effects. Therefore, formulations that have minimum effect on the

Table 1
A single intravenous dose (50 mg/kg CPT-11) of Camptosar® or liposomal irinotecan was used to treat RAG-2M mice bearing established subcutaneous human LS 180 adenocarcinoma colorectal xenografts

Treatment	CPT-11 dose (mg/kg)	Lipid dose (mg/kg)	Time to 400% TGI (days)[a]			Max. mean %BWL (±SEM)[c]
			Mean (±SEM)	Median	Median $T - C$[b]	
Camptosar®	50	n/a	21.8 (1.4)	20.5	0.5	5.7 (1.1)
Liposomal CPT-11 ($CuSO_4$ pH 7.5)	50	225	23.0 (1.2)	23	3	2.5 (0.8)
Liposomal CPT-11 ($CuSO_4$)	50	225	25.3 (1.4)	25	5	7.1 (0.8)
Liposomal CPT-11 ($CuSO_4$ + A23187)	50	225	28.6 (1.3)[d,e]	29	9	6.5 (2.4)
Saline	n/a	n/a	21.8 (1.5)	20	n/a	1.6 (1.3)

[a] The time taken (days) for s.c. LS 180 tumours (~100 mg) to increase 400% in size following a single dose of indicated treatment or control. The results for six animals are represented as the mean ± standard error of the mean (parentheses), or as the median.
[b] The median difference in days for treated (T) tumours to increase in size by 400% compared to saline control tumours (C).
[c] The maximum mean percent body weight loss (%BWL) recorded for each treated/control group ($n = 6$). The standard error of the mean is included in parentheses.
[d] $p < 0.05$ compared to Camptosar®, $CuSO_4$ pH 7.5, saline (see Section 2).
[e] $p < 0.08$ compared to $CuSO_4$ unbuffered (see Section 2).

pharmacokinetics of irinotecan would mediate a $T-C$ value comparable to that of the unencapsulated drug.

The maximum mean percent body weight loss (%BWL) associated with the treatments is shown in Table 1. These data indicate that gains in efficacy afforded by liposome encapsulation of irinotecan were not associated with a concomitant increase in toxicity as judged by %BWL. In contrast to the improvements in efficacy, there was no correlation between improved irinotecan retention and %BWL. Further, the treatment-induced losses in body weight were transient (data not shown).

4. Discussion

The ultimate aim of any drug delivery strategy is to maximise efficacy while minimising toxicity by limiting the bioavailability of the drug until it reaches the target site. Resultantly, efforts have focused on engineering drug carriers, which can both retain the drug and subsequently release it at the desired site of action [49]. A number of technologies have been reported to trigger the release of payloads from liposomes by incorporating lipid components in the liposomal bilayer that react to external stimuli such as pH [50], light [51] or temperature [52]. In this study, we focused on methods for the improved liposomal retention of encapsulated contents following systemic administration. We introduce a novel drug encapsulation technology that was demonstrated to better retain the cytotoxic drug, irinotecan.

DSPC/Chol (55:45 mol%) liposomes with entrapped copper solutions have been reported to efficiently encapsulate irinotecan and topotecan; further, the encapsulated drugs were shown to predominate as the active lactone form [15,53]. These previous studies focused on liposomes with internal solutions of unbuffered 300 mM $CuSO_4$ (initial pH 3.5), 300 mM $CuSO_4$ buffered to pH 7.5, or 100 mM Cu gluconate buffered to pH 7.0 where the formulations were suspended in a neutral external buffer. It was demonstrated that transmembrane copper ion gradients in the presence or absence of an associated pH gradient encapsulated irinotecan with comparable efficiency suggesting an irinotecan–copper interaction [35,54]. Indeed, others have reported that camptothecins can form complexes with transition metals [55,56] and we have demonstrated that copper forms a complex with topotecan [15]. In the present study, the in vivo behaviours of the unbuffered 300 mM $CuSO_4$ (initial pH 3.5) and the 300 mM $CuSO_4$ buffered to pH 7.5 irinotecan formulations were compared to that of a copper-based liposomal preparation of irinotecan where the drug was entrapped in liposomes containing unbuffered 300 mM $CuSO_4$ in the presence of the divalent metal ionophore A23187. The ionophore was used to generate and maintain a transmembrane pH gradient [34].

The rate and extent of active irinotecan encapsulation mediated by the three Cu-based liposome preparations and an $MnSO_4$ plus A23187 formulation were comparable as illustrated in Fig. 1. In contrast, the plasma elimination profiles were distinct (Fig. 2) with the half-life of irinotecan release from the liposome preparations in the order: 300 mM $CuSO_4$ plus A23187 (44.4 h) >300 mM $MnSO_4$ plus A23187 (10.0 h) >300 mM $CuSO_4$ (8.7 h) >300 mM $CuSO_4$ pH 7.5 (5.5 h). The DSPC/Chol (55:45 mol%) $CuSO_4$ plus A23187 formulation mediated a fourfold improvement in the half-life of irinotecan plasma release reported for a comparable irinotecan formulation [54]. These results demonstrated that improved drug retention is dependent on the choice of transition metal and the maintenance of the transmembrane pH gradient (acidic inside). Reports from the laboratory of Pieter Cullis clearly show that when the pH of the liposome interior is low, encapsulated drugs with protonisable amine functions, such as irinotecan, are predominately ionised (i.e., less membrane permeable) and are therefore better retained compared to the same drug encapsulated in an entrapped buffer at a neutral pH [30,40,45]. This behaviour is evident in the drug release rates from the Cu^{2+}-containing liposome formulations. The fastest rate of irinotecan release was observed for liposomes prepared in $CuSO_4$ buffered to pH 7.5, intermediate drug release rates were associated with liposomes prepared in unbuffered $CuSO_4$ where an initial pH gradient (prior to drug addition) collapses following irinotecan loading [35], and the slowest drug release rates were obtained with the irinotecan formulations prepared using $CuSO_4$ plus A23187.

The most surprising aspect of these data, which emphasises the importance of the transition metal, is the fourfold improvement in the half-life of irinotecan release from the Cu^{2+}/A23187 formulation versus the Mn^{2+}/A23187 formulation. It was anticipated that the divalent metal ions would act as "inert" facilitators for the ionophore generated transmembrane pH gradient. However, Fig. 2c suggests that the entrapped metal ions also played a role. We know that Cu^{2+} alone can drive encapsulation of the drug, even in the absence of a pH gradient [35], so it is possible that Cu^{2+} interactions with the drug influence drug retention in these formulations. There is no evidence to suggest that Mn^{2+} alone can promote remote loading of irinotecan or other camptothecins. Interestingly, Dicko et al. [54] investigated the interaction of irinotecan and copper gluconate/TEA in solution at pH 7.0 using Fourier Transformed Infrared Spectroscopy (FT-IR), Circular Dichroism (CD), Raman spectroscopy and Electron Paramagnetic Resonance (EPR) and found no evidence of copper ions binding to irinotecan. In contrast, EPR studies did indicate a change in the EPR signal of $CuSO_4$ with the addition of irinotecan at pH 4.0 [54]. This is consistent with previous EPR studies that indicated copper could interact with the hydroxyl group present in the E ring lactone of camptothecin [55,56]. As indicated previously, our group has recently published evidence showing topotecan can inhibit formation of Cu-hydroxide ions [15], which is consistent with the idea that Cu^{2+} can form a complex with camptothecins.

It should be noted that preliminary atomic absorption spectroscopy assessments of Cu^{2+} have indicated that as much as 70% of the initial entrapped copper ions are shuttled to the exterior of the liposomes during irinotecan loading and following encapsulation it can be estimated that the Cu^{2+} to irinotecan ratio is approximately 1:2. The significance of this ratio remains to be determined. It could indicate that a Cu^{2+} ion interacts with the E ring hydroxyl groups of two irinotecan molecules forming a coordination complex. As a caveat, the majority of studies investigating drug–metal interactions take place in solution at concentrations much lower than that experienced within the aqueous core of liposomes. Cryo-TEM analysis of liposomal irinotecan encapsulated using Cu^{2+}/A23187 did not reveal any drug precipitates (Fig. 4); rather, there was evidence of electron dense intravesicular spots in some liposomes (Fig. 4b). These results are comparable to those of the other Cu^{2+}-containing liposomal irinotecan formulations [35]. It is clear that the improved irinotecan retention afforded by the Cu^{2+}/A23187 formulation is not mediated by the formation of intravesicular precipitates that are detectable by Cryo-TEM analysis. Ongoing studies are continuing to investigate the intravesicular copper–irinotecan interaction, but will also address the potential of copper–lipid interactions to influence drug release [57].

Drug release rates are considered to be the most important factor governing the therapeutic activity of liposomal anti-cancer drugs [38,41]. However, it has been difficult to predict what drug release rate is required for maximum therapeutic activity for any one drug. Therefore, optimisation of a liposomal anti-cancer drug formulation is often determined empirically using animal models. Results obtained for liposomal formulations of cisplatin [58], vincristine [39] and mitoxantrone [42] indicate that when drug release rates are too slow significant reductions in therapeutic effects are observed. Until recently it was believed that decreases in the release of the cell cycle specific drug vincristine would be associated with increasing therapeutic effects; however, formulations have now been adjusted to the point where the rate of vincristine release is so slow that it actually compromises therapeutic activity [41]. The gains in irinotecan retention afforded by the $CuSO_4$ plus A23187 loading methodology were associated with significant improvements in therapeutic efficacy, as judged in an established human LS 180 colorectal xenograft model (Table 1 and Fig. 6). In contrast to the experience with liposome formulations of vincristine and mitoxantrone [41–43], the formulations described here did not retain irinotecan to the extent that a loss in therapeutic activity was observed, suggesting that further gains in drug retention may improve the effectiveness of liposomal irinotecan. Specifically, we will examine the role of liposomal lipid composition on drug retention mediated by the $CuSO_4$ plus A23187 loading methodology. We have recently reported that long-circulating liposomal formulations prepared in the absence of cholesterol can retain certain drugs, e.g., idarubicin [59], much better then cholesterol-containing liposomes. Further, incorporation of sphingomyelin into the liposomal bilayer can facilitate significant increases in drug retention [31,60,61].

The activity of irinotecan is dependent on the maintenance of the closed lactone ring form of the drug; further, irinotecan is metabolised to liberate the potent metabolite SN-38, the activity of which is also dependent on the lactone ring. Fig. 5 demonstrates that irinotecan encapsulated by the Cu^{2+} plus A23187 existed predominately as the active lactone form. This is not unexpected based on the acidic interior of the liposome and is comparable to results obtained for unbuffered $CuSO_4$ and $CuSO_4$ pH 7.5 formulations [35]. The focus of these studies was to investigate the correlation between improved liposomal retention of irinotecan and increased anti-tumour activity against a model of colorectal cancer. Consequently, a full pharmacokinetic characterisation of irinotecan and SN-38 as a function of liposomal encapsulation technology was not undertaken. Nevertheless, the combination of Cu in the presence of A23187, which maintains a transmembrane pH gradient, provides an environment for the improved retention of irinotecan while maintaining the drug in its active form. This is supported by the preliminary in vivo data reported here which clearly shows that the Cu^{2+}/A23187 liposomal formulation of irinotecan is significantly more active than the free drug, a result that is consistent with other formulations with improved irinotecan retention [10,62]. Further, we believe that the success of such a formulation will be dependent on its use in combination with other drugs and we have recently reported on a liposomal formulation containing both irinotecan and floxuridine [53,63]. Our team is currently pursuing development of the optimised liposomal irinotecan formulation as a single agent, with the aim of eventually establishing its therapeutic effects when used in combination with cisplatin, carboplatin or 5-FU/leucovorin-containing treatment regimens.

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References

[1] L. Saltz, Irinotecan-based combinations for the adjuvant treatment of stage III colon cancer, Oncology (Williston Park) 14 (2000) 47–50.
[2] L.B. Saltz, J.V. Cox, C. Blanke, L.S. Rosen, L. Fehrenbacher, M.J. Moore, J.A. Maroun, S.P. Ackland, P.K. Locker, N. Pirotta, G.L. Elfring, L.L. Miller, Irinotecan plus fluorouracil and leucovorin for metastatic colorectal cancer, Irinotecan Study Group, N. Engl. J. Med. 343 (2000) 905–914.
[3] K. Noda, Y. Nishiwaki, M. Kawahara, S. Negoro, T. Sugiura, A. Yokoyama, M. Fukuoka, K. Mori, K. Watanabe, T. Tamura, S. Yamamoto, N. Saijo, Irinotecan plus cisplatin compared with etoposide plus cisplatin for extensive small-cell lung cancer, N. Engl. J. Med. 346 (2002) 85–91.

[4] J.H. Baek, J.G. Kim, S.B. Jeon, Y.S. Chae, D.H. Kim, S.K. Sohn, K.B. Lee, Y.J. Choi, H.J. Shin, J.S. Chung, G.J. Cho, H.Y. Jung, W. Yu, Phase II study of capecitabine and irinotecan combination chemotherapy in patients with advanced gastric cancer, Br. J. Cancer 94 (2006) 1407–1411.
[5] G. Klautke, U. Kuchenmeister, T. Foitzik, K. Ludwig, F. Prall, E. Klar, R. Fietkau, Concurrent chemoradiation with capecitabine and weekly irinotecan as preoperative treatment for rectal cancer: results from a phase I/II study, Br. J. Cancer 94 (2006) 976–981.
[6] Y.H. Hsiang, M.G. Lihou, L.F. Liu, Arrest of replication forks by drug-stabilized topoisomerase I-DNA cleavable complexes as a mechanism of cell killing by camptothecin, Cancer Res. 49 (1989) 5077–5082.
[7] T.G. Burke, Chemistry of the camptothecins in the bloodstream. Drug stabilization and optimization of activity, Ann. N. Y. Acad. Sci. 803 (1996) 29–31.
[8] S.A. Abraham, K. Edwards, G. Karlsson, N. Hudon, L.D. Mayer, M.B. Bally, An evaluation of transmembrane ion gradient-mediated encapsulation of topotecan within liposomes, J. Control. Release 96 (2004) 449–461.
[9] T.H. Chou, S.C. Chen, I.M. Chu, Effect of composition on the stability of liposomal irinotecan prepared by a pH gradient method, J. Biosci. Bioeng. 95 (2003) 405–408.
[10] D.C. Drummond, C.O. Noble, Z. Guo, K. Hong, J.W. Park, D.B. Kirpotin, Development of a highly active nanoliposomal irinotecan using a novel intraliposomal stabilization strategy, Cancer Res. 66 (2006) 3271–3277.
[11] Y.L. Hao, Y.J. Deng, Y. Chen, K.Z. Wang, A.J. Hao, Y. Zhang, In-vitro cytotoxicity, in-vivo biodistribution and anti-tumour effect of PEGylated liposomal topotecan, J. Pharm. Pharmacol. 57 (2005) 1279–1287.
[12] C.L. Messerer, E.C. Ramsay, D. Waterhouse, R. Ng, E.M. Simms, N. Harasym, P. Tardi, L.D. Mayer, M.B. Bally, Liposomal irinotecan: formulation development and therapeutic assessment in murine xenograft models of colorectal cancer, Clin. Cancer Res. 10 (2004) 6638–6649.
[13] A. Pal, S. Khan, Y.F. Wang, N. Kamath, A.K. Sarkar, A. Ahmad, S. Sheikh, S. Ali, D. Carbonaro, A. Zhang, I. Ahmad, Preclinical safety, pharmacokinetics and antitumor efficacy profile of liposome-entrapped SN-38 formulation, Anticancer Res. 25 (2005) 331–341.
[14] M.V. Seiden, F. Muggia, A. Astrow, U. Matulonis, S. Campos, M. Roche, J. Sivret, J. Rusk, E. Barrett, A phase II study of liposomal lurtotecan (OSI-211) in patients with topotecan resistant ovarian cancer, Gynecol. Oncol. 93 (2004) 229–232.
[15] A.S. Taggar, J. Alnajim, M. Anantha, A. Thomas, M. Webb, E. Ramsay, M.B. Bally, Copper–topotecan complexation mediates drug accumulation into liposomes, J. Control. Release 114 (2006) 78–88.
[16] P. Tardi, E. Choice, D. Masin, T. Redelmeier, M. Bally, T.D. Madden, Liposomal encapsulation of topotecan enhances anticancer efficacy in murine and human xenograft models, Cancer Res. 60 (2000) 3389–3393.
[17] T.G. Burke, X. Gao, Stabilization of topotecan in low pH liposomes composed of distearoylphosphatidylcholine, J. Pharm. Sci. 83 (1994) 967–969.
[18] Y.L. Hao, Y.J. Deng, Y. Chen, X.M. Wang, H.J. Zhong, X.B. Suo, In vitro and in vivo studies of different liposomes containing topotecan, Arch. Pharm. Res. 28 (2005) 626–635.
[19] J.J. Liu, R.L. Hong, W.F. Cheng, K. Hong, F.H. Chang, Y.L. Tseng, Simple and efficient liposomal encapsulation of topotecan by ammonium sulfate gradient: stability, pharmacokinetic and therapeutic evaluation, Anticancer Drugs 13 (2002) 709–717.
[20] Y. Sadzuka, S. Hirotsu, S. Hirota, Effective irinotecan (CPT-11)-containing liposomes: intraliposomal conversion to the active metabolite SN-38, Jpn. J. Cancer Res. 90 (1999) 226–232.
[21] D. Subramanian, M.T. Muller, Liposomal encapsulation increases the activity of topoisomerase I inhibitor topotecan, Oncol. Res. 7 (1995) 461–469.
[22] X. Li, W.L. Lu, G.W. Liang, G.R. Ruan, H.Y. Hong, C. Long, Y.T. Zhang, Y. Liu, J.C. Wang, X. Zhang, Q. Zhang, Effect of stealthy liposomal topotecan plus amlodipine on the multidrug-resistant leukaemia cells in vitro and xenograft in mice, Eur. J. Clin. Invest. 36 (2006) 409–418.
[23] S. Lei, P.Y. Chien, S. Sheikh, A. Zhang, S. Ali, I. Ahmad, Enhanced therapeutic efficacy of a novel liposome-based formulation of SN-38 against human tumor models in SCID mice, Anticancer Drugs 15 (2004) 773–778.
[24] V. Peikov, S. Ugwu, M. Parmar, A. Zhang, I. Ahmad, pH-dependent association of SN-38 with lipid bilayers of a novel liposomal formulation, Int. J. Pharm. 299 (2005) 92–99.
[25] J.A. Zhang, T. Xuan, M. Parmar, L. Ma, S. Ugwu, S. Ali, I. Ahmad, Development and characterization of a novel liposome-based formulation of SN-38, Int. J. Pharm. 270 (2004) 93–107.
[26] N.V. Koshkina, E.S. Kleinerman, C. Waldrep, S.-F. Jia, L.L. Worth, B.E. Gilbert, V. Knight, 9-Nitrocamptothecin liposome aerosol treatment of melanoma and osteosarcoma lung metastases in mice, Clin. Cancer Res. 6 (2000) 2876–2880.
[27] K. Gelmon, H. Hirte, B. Fisher, W.V. Walsh, M. Ptaszynski, M. Hamilton, N. Onetto, E.A. Eisenhauer, A phase I study of OSI-211 given as ain intravenous infusion days 1, 2, and 3 every 3 weeks in patients with solid cancers, Invest. New Drugs 22 (2004) 263–275.
[28] C.F. Verschraegen, B.E. Gilbert, A.J. Huaringa, R. Newman, N. Harris, F.J. Leyva, L. Keus, K. Campbell, T. Nelson-Taylor, V. Knight, Feasibility, phase I, and pharmacological study of aerosolized liposomal 9-nitro-20(*S*)-camptothecin in patients with advanced malignancies in the lungs, Ann. N. Y. Acad. Sci. 922 (2000) 352–354.
[29] C.F. Verschraegen, B.E. Gilbert, E. Loyer, A. Huaringa, G. Walsh, R.A. Newman, V. Knight, Clinical evaluation of the delivery and safety of aerosolized liposomal 9-nitro-20(*S*)-camptothecin in patients with advanced pulmonary malignancies, Clin. Cancer Res. 10 (2004) 2319–2326.
[30] P.R. Cullis, M.J. Hope, M.B. Bally, T.D. Madden, L.D. Mayer, D.B. Fenske, Influence of pH gradients on the transbilayer transport of drugs, lipids, peptides and metal ions into large unilamellar vesicles, Biochim. Biophys. Acta 1331 (1997) 187–211.
[31] L. Boehlke, J.N. Winter, Sphingomyelin/cholesterol liposomal vincristine: a new formulation for an old drug, Expert Opin. Biol. Ther. 6 (2006) 409–415.
[32] L.D. Mayer, M.B. Bally, P.R. Cullis, Uptake of adriamycin into large unilamellar vesicles in response to a pH gradient, Biochim. Biophys. Acta 857 (1986) 123–126.
[33] G. Haran, R. Cohen, L.K. Bar, Y. Barenholz, Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases, Biochim. Biophys. Acta 1151 (1993) 201–215.
[34] D.B. Fenske, K.F. Wong, E. Maurer, N. Maurer, J.M. Leenhouts, N. Boman, L. Amankwa, P.R. Cullis, Ionophore-mediated uptake of ciprofloxacin and vincristine into large unilamellar vesicles exhibiting transmembrane ion gradients, Biochim. Biophys. Acta 1414 (1998) 188–204.
[35] E. Ramsay, J. Alnajim, M. Anantha, A. Taggar, A. Thomas, K. Edwards, G. Karlsson, M. Webb, M. Bally, Transition metal-mediated liposomal encapsulation of irinotecan (CPT-11) stabilizes the drug in the therapeutically active lactone conformation, Pharma. Res. 23 (2006) 2799–2808.
[36] T.M. Allen, W.W.K. Cheng, J.I. Hare, K.M. Laginha, Pharmacokinetics and pharmacodynamics of lipidic nano-particles in cancer, AntiCancer Agents Med. Chem. (Formerly Current Medicinal Chemistry - Anti-Cancer Agents) 6 (2006) 513–523.
[37] T.M. Allen, D.R. Mumbengegwi, G.J.R. Charrois, Anti-CD19-targeted liposomal doxorubicin improves the therapeutic efficacy in murine B-cell lymphoma and ameliorates the toxicity of liposomes with varying drug release rates, Clin. Cancer Res. 11 (2005) 3567–3573.
[38] G.J. Charrois, T.M. Allen, Drug release rate influences the pharmacokinetics, biodistribution, therapeutic activity, and toxicity of

pegylated liposomal doxorubicin formulations in murine breast cancer, Biochim. Biophys. Acta 1663 (2004) 167–177.
[39] G.Z. Zhu, E. Oto, Y.P. Quinn, M.S. Newman, C. Engbers, P. Uster, The effect of vincristine-polyanion complexes in STEALTH liposomes on pharmacokinetics, toxicity and anti tumor activity, Cancer Chemother. Pharmacol. 39 (1996) 138–142.
[40] N.L. Boman, L.D. Mayer, P.R. Cullis, Optimization of the retention properties of vincristine in liposomal systems, Biochim. Biophys. Acta 1152 (1993) 253–258.
[41] M.J. Johnston, S.C. Semple, S.K. Klimuk, K. Edwards, M.L. Eisenhardt, E.C. Leng, G. Karlsson, D. Yanko, P.R. Cullis, Therapeutically optimized rates of drug release can be achieved by varying the drug-to-lipid ratio in liposomal vincristine formulations, Biochim. Biophys. Acta 1758 (2006) 55–64.
[42] H.J. Lim, D. Masin, T.D. Madden, M.B. Bally, Influence of drug release characteristics on the therapeutic activity of liposomal mitoxantrone, J. Pharmacol. Exp. Ther. 281 (1997) 566–573.
[43] H.J. Lim, D. Masin, N.L. McIntosh, T.D. Madden, M.B. Bally, Role of drug release and liposome-mediated drug delivery in governing the therapeutic activity of liposomal mitoxantrone used to treat human A431 and LS180 solid tumors, J. Pharmacol. Exp. Ther. 292 (2000) 337–345.
[44] M.J. Hope, M. Bally, L. Mayer, A.S. Janoff, P. Cullis, Generation of multilamellar and unilamellar phospholipid vesicles, Chem. Phys. Lipids 40 (1986) 89–107.
[45] P.R. Harrigan, K.F. Wong, T.E. Redelmeier, J.J. Wheeler, P.R. Cullis, Accumulation of doxorubicin and other lipophilic amines into large unilamellar vesicles in response to transmembrane pH gradients, Biochim. Biophys. Acta 1149 (1993) 329–338.
[46] H.-H. Fiebig, A.M. Burger, in: B.A. Teicher (Ed.), Tumour Models in Cancer Research, Humana Press Inc., Totowa, 2002, pp. 113-137.
[47] M. Bally, L. Mayer, M.J. Hope, R. Nayar, in: G. Gregoriadis (Ed.), Liposome Technology, CRC Press Inc., Boca Raton, 1993, pp. 27-41.
[48] I.V. Zhigaltsev, N. Maurer, K. Edwards, G. Karlsson, P.R. Cullis, Formation of drug-arylsulfonate complexes inside liposomes: a novel approach to improve drug retention, J. Control. Release 110 (2006) 378–386.
[49] D.C. Drummond, O. Meyer, K. Hong, D.B. Kirpotin, D. Papahadjopoulos, Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors, Pharmacol. Rev. 51 (1999) 691–743.
[50] T. Ishida, Y. Okada, T. Kobayashi, H. Kiwada, Development of pH-sensitive liposomes that efficiently retain encapsulated doxorubicin (DXR) in blood, Int. J. Pharm. 309 (2006) 94–100.
[51] R.H. Bisby, C. Mead, A.C. Mitchell, C.G. Morgan, Fast laser-induced solute release from liposomes sensitized with photochromic lipid: effects of temperature, lipid host, and sensitizer concentration, Biochem. Biophys. Res. Commun. 262 (1999) 406–410.
[52] D. Needham, G. Anyarambhatla, G. Kong, M.W. Dewhirst, A new temperature-sensitive liposome for use with mild hyperthermia: characterization and testing in a human tumor xenograft model, Cancer Res. 60 (2000) 1197–1201.
[53] P.G. Tardi, R.C. Gallagher, S. Johnstone, N. Harasym, M. Webb, M.B. Bally, L.D. Mayer, Coencapsulation of irinotecan and floxuridine into low cholesterol-containing liposomes that coordinate drug release in vivo, Biochim. Biophys. Acta (BBA) – Biomembr. 1768 (2007) 678–687.
[54] A. Dicko, P. Tardi, X. Xie, L. Mayer, Role of copper gluconate/triethanolamine in irinotecan encapsulation inside the liposomes, Int. J. Pharm. 337 (2007) 219–228.
[55] V. Brezova, M. Valko, M. Breza, H. Morris, J. Telser, D. Dvoranova, K. Kaiserova, L. Varecka, M. Mazur, D. Leibfritz, Role of radicals and singlet oxygen in photoactivated dna cleavage by the anticancer drug camptothec: an electron paramagnetic resonance study, J. Phys. Chem. B 107 (2003) 2415–2425.
[56] J. Kuwahara, T. Suzuki, K. Funakoshi, Y. Sugiura, Photosensitive DNA cleavage and phage inactivation by copper(II)–camptothecin, Biochemistry 25 (1986) 1216–1221.
[57] M. Suwalsky, B. Ungerer, L. Quevedo, F. Aguilar, C.P. Sotomayor, Cu^{2+} ions interact with cell membranes, J. Inorg. Biochem. 70 (1998) 233–238.
[58] S. Bandak, D. Goren, A. Horowitz, D. Tzemach, A. Gabizon, Pharmacological studies of cisplatin encapsulated in long-circulating liposomes in mouse tumor models, Anticancer Drugs 10 (1999) 911–920.
[59] N. Dos Santos, L.D. Mayer, S.A. Abraham, R.C. Gallagher, K.A. Cox, P.G. Tardi, M.B. Bally, Improved retention of idarubicin after intravenous injection obtained for cholesterol-free liposomes, Biochim. Biophys. Acta 1561 (2002) 188–201.
[60] M.S. Webb, P. Logan, P.M. Kanter, G. St. Onge, K. Gelmon, T. Harasym, L.D. Mayer, M.B. Bally, Preclinical pharmacology, toxicology and efficacy of sphingomyelin/cholesterol liposomal vincristine for therapeutic treatment of cancer, Cancer Chemother. Pharmacol. 42 (1998) 461–470.
[61] I.V. Zhigaltsev, N. Maurer, Q.F. Akhong, R. Leone, E. Leng, J. Wang, S.C. Semple, P.R. Cullis, Liposome-encapsulated vincristine vinblastine and vinorelbine: a comparative study of drug loading and retention, J. Control. Release 104 (2005) 103–111.
[62] C.O. Noble, M.T. Krauze, D.C. Drummond, Y. Yamashita, R. Saito, M.S. Berger, D.B. Kirpotin, K.S. Bankiewicz, J.W. Park, Novel nanoliposomal CPT-11 infused by convection-enhanced delivery in intracranial tumors: pharmacology and efficacy, Cancer Res. 66 (2006) 2801–2806.
[63] L.D. Mayer, T.O. Harasym, P.G. Tardi, N.L. Harasym, C.R. Shew, S.A. Johnstone, E.C. Ramsay, M.B. Bally, A.S. Janoff, Ratiometric dosing of anticancer drug combinations: controlling drug ratios after systemic administration regulates therapeutic activity in tumor-bearing mice, Mol. Cancer Ther. 5 (2006) 1854–1863.