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Coencapsulation of irinotecan and floxuridine into low cholesterol-containing liposomes that coordinate drug release in vivo

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Abstract

A liposomal delivery system that coordinates the release of irinotecan and floxuridine in vivo has been developed. The encapsulation of floxuridine was achieved through passive entrapment while irinotecan was actively loaded using a novel copper gluconate/triethanolamine based procedure. Coordinating the release rates of both drugs was achieved by altering the cholesterol content of distearoylphosphatidylcholine (DSPC)/distearoylphosphatidylglycerol (DSPG) based formulations. The liposomal retention of floxuridine in plasma after intravenous injection was dramatically improved by decreasing the cholesterol content of the formulation below 20 mol%. In the case of irinotecan, the opposite trend was observed where increasing cholesterol content enhanced drug retention. Liposomes composed of DSPC/DSPG/Chol (7:2:1, mole ratio) containing co-encapsulated irinotecan and floxuridine at a 1:1 molar ratio exhibited matched leakage rates for the two agents so that the 1:1 ratio was maintained after intravenous administration to mice. The encapsulation of irinotecan was optimal when copper gluconate/triethanolamine (pH 7.4) was used as the intraliposomal buffer. The efficiency of irinotecan loading was approximately 80% with a starting drug to lipid molar ratio of 0.1/1. Leakage of floxuridine from the liposomes during irinotecan loading at 50 °C complicated the ability to readily achieve the target 1:1 irinotecan/floxuridine ratio inside the formulation. As a result, a procedure for the simultaneous encapsulation of irinotecan and floxuridine was developed. This co-encapsulation method has the advantage over sequential loading in that extrusion can be performed in the absence of chemotherapeutic agents and the drug/drug ratios in the final formulation can be more precisely controlled.

Keywords: Liposomes; Irinotecan; Floxuridine; Coordinated release; Metal loading

1. Introduction

Liposomes have been used extensively to improve the therapeutic index of a variety of drugs by ameliorating toxicity and/or increasing the therapeutic potency of the encapsulated agent [1,2]. This is perhaps best exemplified in the delivery of anticancer drugs where it has been well documented both preclinically and clinically that small (approximately 100 nm) liposomes reduce exposure of entrapped drugs to susceptible healthy tissues while preferentially accumulating in sites of tumor growth due to enhanced permeability and retention (EPR) effects associated with solid tumors [3–6]. This in turn has often resulted in improvements of the overall therapeutic activity of the drug and has led to the regulatory approval of several liposome-based anticancer products [7,8]. Interestingly, very little work has been undertaken to deliver drug combinations in liposomes. This is likely the result of difficulties associated with the efficient and stable encapsulation of two chemotherapeutics inside a single liposome as well as challenges in controlling the release of chemically disparate drugs with one liposome composition.

Our interest in developing liposome formulations containing co-encapsulated anticancer drug combinations stems from the fact that virtually all curative cancer treatment regimens utilize drug combinations. We hypothesized that enhanced antitumor activity may be achieved by simultaneously delivering and

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exposing anticancer drug combinations to tumor cells in vivo, since tumor cells would be less able to develop compensatory resistance mechanisms compared to single or sequentially administered agents. This approach became of even greater importance when we recently observed that the antitumor activity of drug combinations can be dramatically dependent on the molecular ratio of the combined drugs [9]. Specifically, some ratios of a drug combination can be synergistic whereas other ratios of the same drugs can be additive or even antagonistic [10]. This highlighted the need to control drug ratios being exposed to tumor cells after systemic administration since the uncontrolled and dissimilar pharmacokinetics of individual drugs utilized in conventional drug "cocktails" no doubt results in exposure to sub-optimal drug ratios with a concomitant loss in therapeutic activity. Further, although an increasing number of individual liposomal anticancer drugs are being developed, the pharmacokinetics of currently available formulations vary greatly which would hinder attempts to coordinate the exposure of different drugs using existing liposome formulations [11,12]. In addition, combining multiple liposome-based anticancer drugs poses potential difficulties due to high lipid doses which have been shown in humans to lead to infusion-related adverse events [13,14].

In view of the above considerations, we undertook a series of studies aimed at developing single liposome formulations that are able to stably co-encapsulate drug combinations and coordinate the release of the two agents after intravenous (i.v.) injection. In this report we describe the co-encapsulation of irinotecan and floxuridine. This drug combination was selected based on the fact that irinotecan and fluoropyrimidine (typically 5-fluorouracil, 5-FU) combination treatment is standard of care for metastatic colorectal cancer. The focus on floxuridine rather than 5-FU was based on the superior encapsulation and retention properties of floxuridine compared to 5-FU [15] and the fact that trials conducted in the 1960s established the clinical equivalency of these two fluoropyrimidines [16,17]. We have shown in previous studies that a 1:1 molar ratio of these drugs optimizes drug synergy in a panel of gastrointestinal tumor lines [9]. We describe here the identification of liposome encapsulation techniques and lipid compositions that result in stable drug entrapment and drug release rates that maintain irinotecan and floxuridine at a 1:1 molar ratio after i.v. administration.

2. Materials and methods

2.1. Lipids, drugs and chemicals

Distearoylphosphatidylglycerol (DSPG), distearoylphosphatidylcholine (DSPC) and cholesterol were obtained from Avanti Polar Lipids (Alabaster, AL, USA). Irinotecan hydrochloride trihydrate (Camptosar) is a product of Pharmacia and Upjohn Company (Kalamazoo, MI, USA) and was obtained from the pharmacy of the British Columbia Cancer Agency. It was also obtained as a dry powder from ScinoPharm Taiwan, Ltd. (Tainan, Taiwan). Floxuridine was obtained from the Zhejiang Hisun Pharmaceutical Company (Taizhou City, China). Radiolabelled [^{3}H]floxuridine was obtained from Moravek Biochemicals (Brea, CA, USA). Cholesterol hexadecyl ether (CHE) radiolabelled with [^{3}H] or [^{14}C] was obtained from NEN Life Science Products (Oakville, ON, Canada). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA).

2.2. Preparation of liposomes

Based on the appropriate molar composition, DSPC and DSPG were dissolved in chloroform/methanol/water (5/1/.1) at 50 mg/ml and combined with cholesterol dissolved in chloroform. Where appropriate, cholesteryl hexadecyl ether [^{14}C]CHE or [^{3}H]CHE was added as a non-exchangeable, non-metabolizable lipid marker [18]. The solvent was removed under a stream of nitrogen gas and the lipid films placed under high vacuum overnight. Typically, the lipid films were rehydrated at 70 °C with 100 mM copper gluconate, 220 mM triethanolamine (TEA), pH 7.4. The pH of the copper gluconate solution was adjusted by varying the TEA content and not through the addition of sodium hydroxide. In experiments involving the sequential loading of floxuridine and irinotecan, the floxuridine was passively trapped in the liposomes by adding 122 mM floxuridine plus [^{3}H]floxuridine as a tracer to the copper gluconate buffer. The newly formed multilamellar vesicles (MLVs) were passed 10 times through an extruding apparatus (Northern Lipids) containing two stacked 100 nm polycarbonate filters. The mean diameter and size distribution of each liposome preparation, analyzed by a NICOMP Model 270 Submicron particle sizer (Pacific Scientific, Santa Barbara, CA, USA) operating at 632.8 nm, was typically 110±20 nm. Following extrusion the external liposomal buffer was exchanged for 300 mM sucrose, 20 mM HEPES, 30 mM EDTA, pH 7.4 (SHE) using tangential flow chromatography.

2.3. Time course of irinotecan encapsulation

The following conditions were used to examine irinotecan encapsulation into liposomes containing various metal salts, copper gluconate at different pH and irinotecan to lipid ratios. Irinotecan (Camptosar) and liposomes were heated separately at 50 °C for 1 min and then combined at $t=0$ by adding the lipid to the drug while vortexing. The final lipid concentration was diluted to 30 mM using SHE buffer. Aliquots of 100 μl were removed at various time points and applied to 1 ml Sephadex G-50 spin columns. The columns were prepared by adding glass wool to a 1 ml syringe and Sephadex G-50 beads hydrated in HEPES buffered saline (HBS; 20 mM HEPES, 150 mM NaCl, pH 7.4). The columns were packed by spinning at 500×g for 2 min. Following addition of the sample to the column, the liposome fraction was collected in the void volume by centrifuging at 650×g for 1 min. Aliquots of the spin column eluant and the pre-column solution were taken and analyzed by liquid scintillation counting to determine the lipid concentration at each time point. The irinotecan concentration in each liposomal fraction was determined using a UV based assay. Briefly, a 100 μl aliquot of each liposomal sample (or smaller volume adjusted to 100 μl with distilled water) was solubilized in 100 μl of 10% Triton X-100 plus 800 μl of 50 mM citrate/trisodium citrate, 15 mM EDTA, pH 5.5 and heated in boiling water until the cloud point was reached. The samples were cooled to room temperature and the absorbance at 370 nm measured and compared to a standard curve. For mouse studies utilizing irinotecan containing formulations, the unencapsulated irinotecan and EDTA was removed by replacing the external buffer with SH buffer (300 mM sucrose, 20 mM HEPES, pH 7.4) using tangential flow chromatography.

2.4. Simultaneous encapsulation of irinotecan and floxuridine

A lipid film composed of DSPC/DSPG/Chol (7:2:1, mol/mol/mol) was prepared as previously described in Section 2.2 containing [^{14}C]CHE as the lipid marker. The film was hydrated with 100 mM copper gluconate, 220 mM TEA, pH 7.4 at 70 °C and the resulting MLVs were extruded at 70 °C through two stacked 100 nm filters for a total of ten passes. The liposomes were subsequently exchanged for SHE using tangential flow chromatography and concentrated to 60 mg/ml. To prepare a drug solution containing floxuridine and irinotecan, ^{3}H-floxuridine was first added to a test tube and dried under a stream of nitrogen. Subsequently, floxuridine and irinotecan were added to the same tube as a powder and hydrated with SHE buffer. The pH of the drug solution was adjusted to 7.4 with NaOH. The amount of irinotecan in the loading solution was based on a 0.12 drug to lipid molar ratio. The final floxuridine concentration was adjusted so that a concentration of 122 mM would be achieved after its addition to the liposome solution. The drug solution and the liposomes were incubated separately at 50 °C for approximately 5 min to equilibrate the temperature. The

two solutions were then combined and 100 μL aliquots were removed at various time points and applied to a Sephadex G-50 spin column as previously described. The drug to lipid ratio for floxuridine, from the eluted column sample, was determined using dual label liquid scintillation counting. The irinotecan concentration was determined by measuring the irinotecan absorbance at 370 nm as previously described.

2.5. Mice

Female Balb/c mice (6–8 weeks), 20–22 g breeders were purchased from Charles River Laboratories (St. Constant, PQ, Canada) and bred in house. Mice were housed in microisolator cages and given free access to water and food. The animals were maintained according to the procedures established at the BC Cancer Agency Joint Animal Facility. All animal studies were approved by the University of British Columbia Animal Care Committee in accordance with the guidelines established by the Canadian Council of Animal Care.

2.6. Plasma elimination of liposomes

To determine the retention of irinotecan and floxuridine in various liposomal formulations in vivo, plasma elimination studies were performed. Radiolabelled liposomes containing floxuridine and/or irinotecan were administered at the appropriate concentration intravenously into the lateral tail vein of mice. At 1, 4 and 24 h after administration, mice were asphyxiated with CO_2 and the blood collected by cardiac puncture and placed into EDTA coated microtainer tubes. The blood was centrifuged at 900×*g* for 10 min at 4 °C to isolate the plasma fraction. Liposomes and floxuridine concentrations were determined by liquid scintillation counting while irinotecan concentrations were determined by HPLC.

2.7. Irinotecan quantitation using HPLC

To determine the $T=0$ irinotecan concentration in the liposome sample prior to injection, the liposomes were diluted 1:100 in saline. Subsequently, a 100 μL aliquot of the diluted liposome sample was mixed with internal standard (camptothecin), followed by adding 600 μL acidified methanol (pH 2.0). For determination of irinotecan HCl in plasma, 10 μL (1-h and 4-h time points) or 20 μL (24-h time points) mouse plasma was mixed with 10 μL internal standard (camptothecin) followed by adding 600 μL acidified methanol (pH 2.0). Samples were then vortexed vigorously and stored in −70 °C freezer for 1 h. After cooling, the samples were centrifuged for 10 min at 1500×*g* and the supernatant was analyzed for irinotecan HCl using HPLC. The chromatographic system consisted of a Waters Alliance 2695 system and 2475 fluorescence detector. Waters Symmetry reverse phase C18 column (4.6×250 mm, 5μ) protected by a Symmetry Sentry guard column (3.9×20 mm, 5μ) was used for chromatographic separation. The analytical column was maintained at a temperature of 30 °C. The mobile phase was composed of acetonitrile −75 mM ammonium acetate containing 7.5 mM tetrabutylammonium bromide (24:76, v/v), with pH adjusted to 6.4 using glacial acetic acid. The mobile phase was delivered isocratically at a flow rate of 1.5 mL/min. Column elute was monitored fluorimetrically at excitation wavelength of 362 nm and emission wavelength of 425 nm. Detection and integration of chromatographic peaks was performed by Waters Empower software. Irinotecan HCl concentration was calculated using a calibration curve (linear range 2–100 μg/mL).

3. Results

3.1. Irinotecan encapsulation into transition metal containing liposomes

Based on the structure of irinotecan and the documented ability of transition metals to interact with drugs [19], we hypothesized that the presence of a free hydroxyl on the E-ring adjacent to the lactone group could be a candidate for the formation of a coordination complex with metal ions [20,21], thus enabling active loading of the irinotecan into preformed liposomes containing a metal salt solution. To determine if irinotecan could be encapsulated into liposomes using transition metals, DSPC/Chol (55:45 mol/mol) liposomes were prepared containing 300 mM sulfate salts of manganese, zinc, copper, nickel or cobalt. The liposomes were incubated with irinotecan at 50 °C using a drug to lipid ratio of 0.1:1 (mol/mol) for 1 h. This temperature was selected based on the fact that temperatures ≤40 °C provided very slow irinotecan encapsulation rates (data not shown). At various time points, aliquots were removed and the encapsulated drug to lipid ratio was determined (Fig. 1). Less than 10% encapsulation was observed under these conditions for all metals except zinc and copper which rapidly accumulated irinotecan. Zinc encapsulation was less efficient than copper with a maximum irinotecan to lipid ratio of 0.075:1 (mol/mol) and showed a gradual decrease in trapped irinotecan over the 60-min time course. Irinotecan encapsulation into copper containing liposomes was very effective with >95% encapsulation efficiency at all time points tested. It should be noted that although evidence of transition metal–fluoropyrimidine interactions have been reported [22], attempts to actively encapsulate floxuridine into metal containing liposomes in a manner similar to irinotecan were unsuccessful (data not shown).

Numerous copper salts (chloride, sulfate, nitrate, gluconate and tartrate) are commercially available, however, only the sulfate and gluconate forms were found to be sufficiently soluble and stable to promote efficient irinotecan encapsulation described here. When dissolved in water, both the sulfate and gluconate forms of copper are acidic with a pH of approximately 4. Since it was desirable to have an internal liposomal pH near neutrality, the copper solutions were adjusted to pH 7.4. When copper sulfate was pH adjusted with sodium hydroxide, however, a significant copper oxide

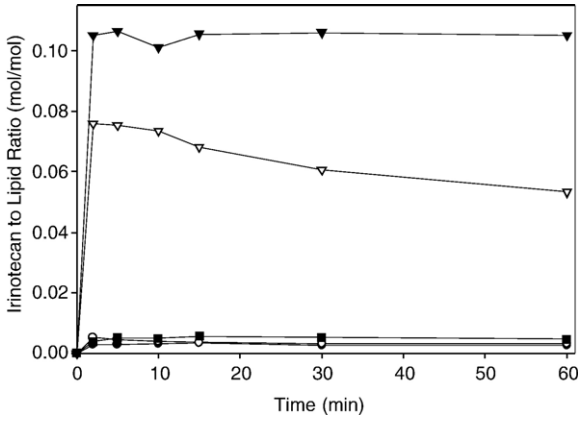

Fig. 1. Irinotecan encapsulation into DSPC/Chol (55:45, mol%) liposomes using various metal salts. Liposomes radiolabelled with [^{3}H]CHE were extruded in the presence of various unbuffered metal salt solutions and subsequently exchanged into SHE buffer (pH 7.4). The liposomes were incubated at 50 °C in the presence of irinotecan at a 0.1/1 molar drug to lipid ratio. At various time points aliquots were removed and assayed for drug encapsulation as outlined in Section 2.3. The following metal solutions, $CuSO_4$ (▼); $ZnSO_4$ (▽); $MnSO_4$ (■); $CoSO_4$ (●) and $NiSO_4$ (○) were all prepared at a concentration of 300 mM and were not pH adjusted (representative data shown).

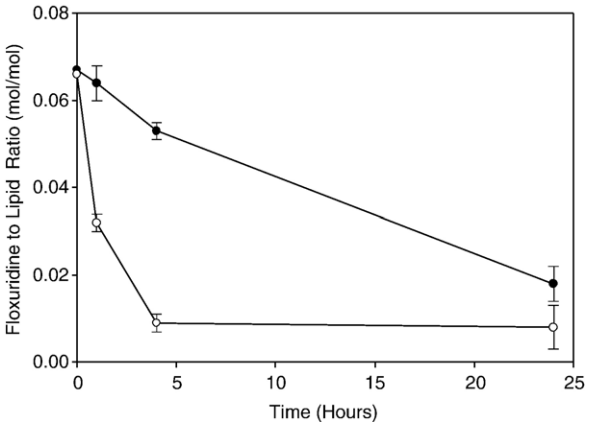

Fig. 2. The influence of liposome composition on the retention of floxuridine in vivo. Liposomes composed of either DSPC/DSPG (8:2 molar ratio, ●) or DSPC/Chol (55: 45 molar ratio, ○) were radiolabelled with [^{14}C]CHE were extruded in the presence of a 100 mM solution of [^{3}H]floxuridine dissolved in HBS (pH 7.4). The unencapsulated floxuridine was exchanged for saline using tangential flow chromatography. The liposomes were injected into the tail vein of female Balb/c mice at a floxuridine dose of 5 mg/kg. Blood was collected via cardiac puncture at 1, 4 and 24 h after injection (3 mice per time point). Lipid and floxuridine recovery was determined by liquid scintillation counting.

precipitate formed immediately after mixing. The biological buffer triethanolamine (TEA) was found to be the most compatible buffer for both copper sulfate and copper gluconate pH adjustment. When pH 7.4 solutions of copper gluconate and copper sulfate were compared for their ability to encapsulate irinotecan, the copper gluconate solution was found to be superior in total capacity, percent encapsulation and drug retention (data not shown). As a result, all subsequent formulation studies focused on copper gluconate buffered with TEA. It should be noted that stable liposome formulations containing gluconate salts of the other transition metals buffered to physiological pH could not be prepared due to instability of the salt solution which lead to significant precipitation (data not shown).

3.2. Floxuridine encapsulation and retention in liposomes

Since floxuridine could not be actively loaded into liposomes via metal complexation, the drug was passively encapsulated into liposomes by hydrating lipid films in the presence of floxuridine solutions. A previous study reported that the trapping efficiency of floxuridine can be increased when trapped in negatively charged liposomes [15]. Also, recent studies have shown that cholesterol-free liposomes can enhance the retention properties of certain drugs [23]. Taken together, this information led us to investigate the in vivo drug retention properties of cholesterol-free, negatively charged liposomes composed of DSPC/DSPG (80:20, mol/mol) compared to DSPC/Chol (55:45, mol/mol) formulations. As shown in Fig. 2, the retention of floxuridine (reflected by maintenance of the encapsulated drug to lipid ratio) inside liposomes after i.v. injection to mice was found to be superior in the cholesterol free formulation. The half life for the release of floxuridine from the liposomes increased from approximately 1 h for DSPC/Chol (55:45, mol/mol) liposomes to approximately 16 h for the DSPC/DSPG (80:20, mol/mol) formulation. Based on the superior floxuridine retention properties and circulation lifetimes, the DSPC/DSPG formulation was studied as a potential delivery system for the drug combination of irinotecan and floxuridine.

3.3. Co-formulation of irinotecan and floxuridine

Initial studies on the co-formulation of irinotecan and floxuridine focused on the hydration of lipid films with solutions of copper gluconate/TEA that contained floxuridine. However, irinotecan encapsulation was not affected by the presence or absence of floxuridine within the liposomes. As a result, the optimization of irinotecan loading was determined in the absence of entrapped floxuridine. The liposomes hydrated from lipid films were extruded and the unencapsulated copper was removed by tangential flow dialysis. The conditions that were found to impact the subsequent encapsulation of irinotecan to the greatest degree were pH and temperature. To determine the optimal pH for irinotecan loading, liposomes were prepared with matching internal and external buffer pH. The time course for irinotecan loading into liposomes at 50 °C is presented in Fig. 3. Maximum irinotecan encapsulation leading to a final drug to lipid ratio of approximately 0.8:1 (mol/mol) was achieved with buffers at pH 7.0 and 7.5. This reflected an encapsulation efficiency of 80% based on the starting irinotecan to lipid ratio of 0.1:1 (mol/mol). The rate of irinotecan accumulation inside the copper containing liposomes was most rapid at pH 7.5 where uptake levels were 85% of maximum within 5 min. Decreasing the pH to 6.5 or 6.0 resulted in decreased rates of irinotecan accumulation and liposome uptake levels after incubating for 60 min were 0.061 and 0.039 μmole drug/μmol lipid, respectively. Due to the similar

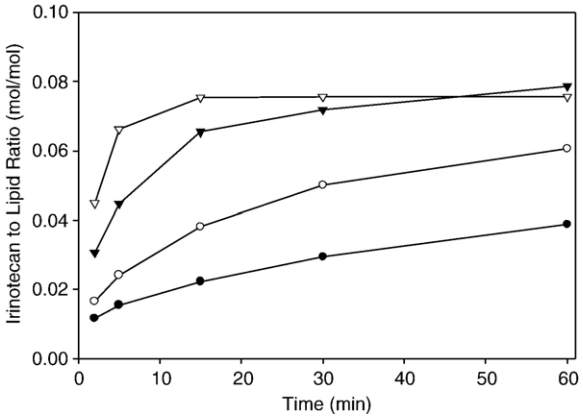

Fig. 3. The effect of pH on irinotecan encapsulation. Liposomes composed of DSPC/DSPG (80:20 molar ratio) were radiolabelled with [^{3}H]CHE and extruded in the presence of 100 mM copper gluconate that was pH adjusted with TEA to pH 7.5 (▽); pH 7 (▼); pH 6.5 (○) or pH 6 (●). The external liposomal solution was replaced with 150 mM NaCl, 10 mM HEPES and 10 mM MES which was pH adjusted to match the internal liposomal pH. Irinotecan was incubated with the liposomes at 50 °C and at various times aliquots were removed and passed through a spin column to determine the extent of irinotecan encapsulation. The irinotecan to lipid ratio was determined as described previously in Section 2.3 (representative data shown).

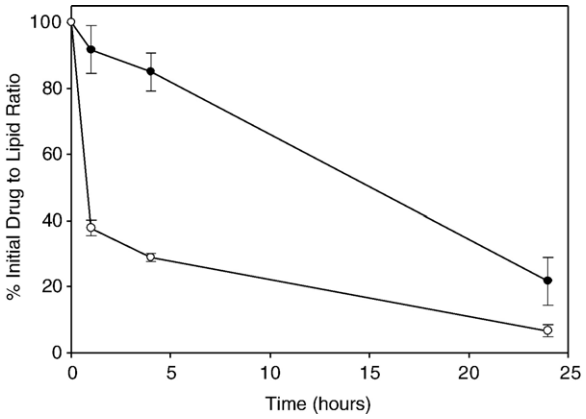

Fig. 4. The in vivo retention of floxuridine and irinotecan coformulated in DSPC/DSPG (80:20 molar ratio) liposomes. Liposomes were radiolabelled with [^{14}C]CHE and extruded in the presence of 100 mM copper gluconate/TEA pH 7.4 containing [^{3}H] labelled floxuridine. The external buffer was exchanged for SHE (pH 7.4) and loaded with irinotecan at a drug to lipid molar ratio of 0.1/1. The unencapsulated irinotecan and EDTA was removed by exchanging into SH buffer using tangential flow chromatography. Mice were injected via the tail vein at a lipid dose of 200 μmol/kg (16 μmol/kg of irinotecan and floxuridine). Blood was recovered at 1, 4 and 24 h after injection and centrifuged to isolate plasma (3 mice per time point). Lipid and floxuridine (●) levels were determined by liquid scintillation counting while irinotecan (○) concentrations were determined by HPLC analysis.

irinotecan loading properties at pH 7 and 7.5, the physiological pH of 7.4 was chosen for all subsequent studies.

Liposomes composed of DSPC/DSPG (80:20, mol/mol), containing both floxuridine and irinotecan at a molar drug ratio of 1:1 (drug to lipid molar ratios of approximately 0.08:1), were administered i.v. into mice to determine if this formulation could coordinate drug release after injection. The drug to lipid ratio for both drugs was monitored in the plasma over 24 h as an indicator of drug release from the liposomes in the circulation. As shown in Fig. 4, the retention of floxuridine in this cholesterol-free formulation was far greater than that observed for irinotecan. The floxuridine drug to lipid ratio decreased linearly over time with a half life of approximately 14 h and 22% of the original drug to lipid ratio remained after 24 h. In contrast, the retention of irinotecan was poor with greater than 60% drug leakage within the first hour after injection and the plasma irinotecan to lipid ratio at 24 h was only 5% of the starting drug to lipid ratio. This large difference in drug release rates lead to circulating irinotecan/floxuridine ratios that rapidly deviated from the desired 1:1 ratio. It should be noted that the rates of irinotecan and floxuridine release when co-formulated inside the same liposome were similar to those obtained for each respective drug encapsulated individually inside the liposomes (data not shown).

In order to better coordinate the release of irinotecan and floxuridine co-formulated inside 100 nm liposomes, an iterative variation of lipid composition was performed. Since cholesterol content was shown to have a dramatic impact on the retention of floxuridine (Fig. 2), we reasoned that it may also play a role in the retention of irinotecan. Various liposome formulations containing cholesterol at levels ranging from 0 to 15% were tested for drug retention in vivo. At 1, 4 and 24 h after injection, blood was collected and the plasma was analyzed for irinotecan, floxuridine and lipid levels. Irinotecan to lipid ratios were found to be significantly affected by cholesterol content (Fig. 5, top panel). In the formulations containing 0 or 5% cholesterol, rapid leakage of irinotecan is observed. One hour after injection of these formulations, the circulating liposomes retained only 38%

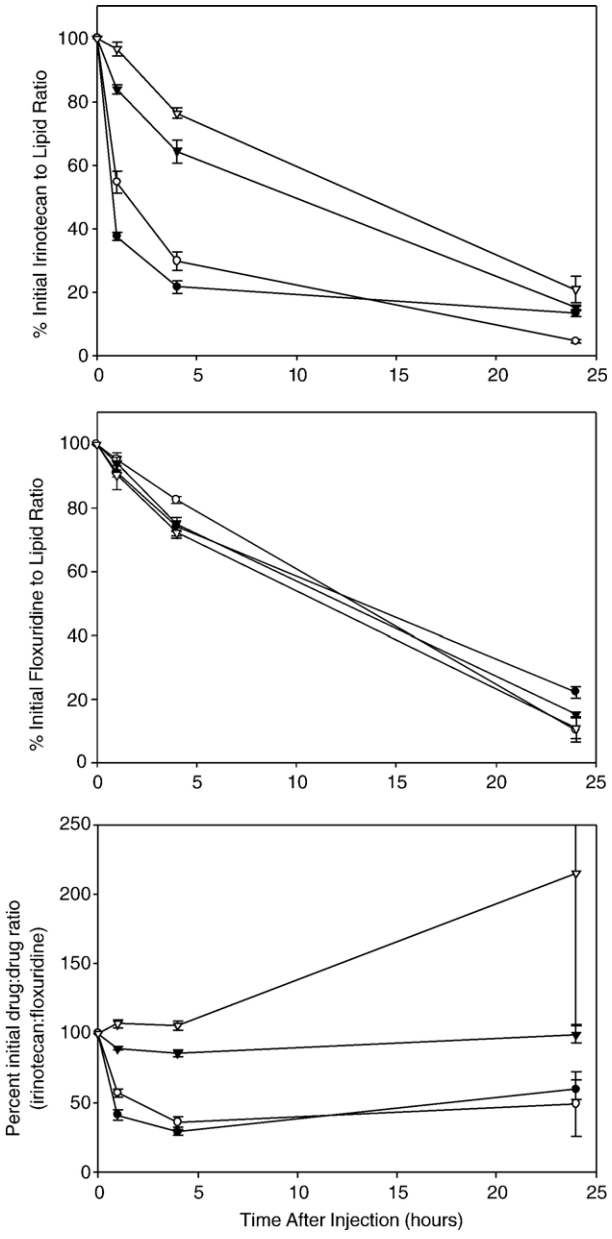

Fig. 5. The in vivo retention of floxuridine and irinotecan coformulated in various liposomal formulations. Liposomes composed of DSPC/Chol/DSPG (65:15:20 molar ratio); ▽, DSPC/Chol/DSPG (70:10:20 molar ratio); ▼, DSPC/Chol/DSPG (75:5:20 molar ratio); ○, and DSPC/DSPG (80:20 molar ratio); ● were radiolabelled with [^{14}C]CHE and coformulated with irinotecan and floxuridine as previously described. Samples were injected into the tail vein of mice at a lipid dose of 370 μmoles/kg (37 μmoles/kg irinotecan and floxuridine). At 1, 4 and 24 h after injection blood was collected and assayed for lipid, irinotecan and floxuridine levels (3 mice per time point). Changes to irinotecan to lipid levels with time are monitored in the upper panel while the middle panel plots the floxuridine to lipid levels from the same formulation. The lower panel charts changes in the drug: drug molar ratio in the various liposomal formulations over time.

and 55% of the initial encapsulated irinotecan, respectively. When the cholesterol content was increased to 10 and 15 mol%, we observed 84 and 97% of initial irinotecan to lipid ratios in the plasma after 1 h. The small increase in cholesterol content from 5 to 10 mol% increased the irinotecan half life by approximately 6.5-fold from 1.5 h to 10 h. This large dependency of irinotecan retention in vivo on cholesterol content was not observed at liposome cholesterol levels above 10 mol%. The sensitivity of irinotecan retention in the 10 mol% cholesterol range was subsequently investigated with formulations containing 7.5, 10 and 12.5 mol% cholesterol. Analysis of irinotecan release rates over 24 h found no significant differences between these three formulations (data not shown). As a result it appears that irinotecan retention is not correlated with cholesterol content in a linear fashion but requires a minimum threshold level. In addition, it should be noted that irinotecan can exist in an open ring carboxylate or closed ring lactone form. Only the lactone form of the drug is biologically active and was found to be the predominant form (>90%) in all the liposomal formulations tested.

The release of floxuridine from DSPC/DSPG-based liposomes as a function of cholesterol content was monitored and the results are presented in Fig. 5 (middle panel). Unlike irinotecan, floxuridine retention was not significantly influenced by cholesterol content between 0 and 15 mol%. The drug to lipid half life for floxuridine ranged from a high of approximately 13 h in the cholesterol free formulation to a low of approximately 11 h in the 15 mol% formulation. This was somewhat surprising since the results shown in Fig. 2 suggest that floxuridine leakage is dramatically enhanced in the presence of 45 mol% cholesterol. The fact that little difference was observed between 0 and 15 mol% cholesterol suggests that, similar to irinotecan leakage, there appears to be a threshold amount of cholesterol that is required before large changes in drug release are observed. When the encapsulated irinotecan to floxuridine molar ratio was plotted (Fig. 5, lower panel) for the 0 and 5 mol% formulations, the starting drug to drug molar ratio was observed to drop from 1:1 to 1:2 within 1 h. The initial drop in the molar drug ratio is the result of rapid leakage of irinotecan from these formulations. Following the initial drop at 1 h, the molar drug ratio is maintained out to 24 h. In the formulation containing 15 mol% cholesterol, the opposite trend was observed where the drug to drug ratio increases as a result of enhanced irinotecan retention relative to floxuridine. The synergistic 1:1 molar drug ratio was optimally maintained in the formulation containing 10 mol% cholesterol for which the release rates of both agents was matched.

3.4. Optimization of drug encapsulation

The studies described thus far have demonstrated that irinotecan and floxuridine can be stably co-encapsulated at a 1:1 molar ratio inside DSPC/DSPG/Chol (70:20:10, mol/mol) liposomes containing copper gluconate/TEA pH 7.4 and that these liposomes coordinate the release of the two drugs, thereby maintaining the irinotecan/floxuridine ratio for extended times after i.v. administration. In an attempt to increase the liposomal drug capacity, copper gluconate/TEA pH 7.4 containing liposomes were incubated at elevated starting irinotecan to lipid ratios to determine the maximum amount of drug that could be loaded into these formulations. Irinotecan encapsulation was not affected by the presence or absence of floxuridine within the liposomes, therefore irinotecan loading was determined in the absence of entrapped drug. At a starting drug to lipid ratio of 0.1:1, approximately 80% drug encapsulation is observed (Fig. 6). A similar encapsulation efficiency was observed at a starting irinotecan to lipid molar ratio of 0.2:1 where the final encapsulated drug to lipid ratio was approximately 0.15:1. However, when the starting irinotecan to lipid ratio was elevated to 0.3:1 the encapsulated irinotecan to lipid ratio increased only to 0.18:1, reflecting a decrease in encapsulation efficiency to approximately 60%. As a result, incubating the liposomes with irinotecan at a 0.2:1 ratio provided the highest drug to lipid ratio in the final formulation (0.14–0.15/1) without significantly decreasing the irinotecan loading efficiency.

Floxuridine levels were also increased in the liposome formulations in order to compensate for the increase in irinotecan to lipid ratio and maintain the 1:1 molar drug ratio inside the liposomes. This was achieved by extruding the liposomes in the presence of elevated floxuridine concentrations. However, floxuridine leakage during irinotecan encapsulation at 50 °C when the higher drug to lipid ratios were utilized caused significant difficulty in controlling final irinotecan to floxuridine ratios in the liposomes. This effect is reflected in Fig. 7 which presents the encapsulated floxuridine to lipid ratio over time for liposomes incubated at temperatures ranging from 40 °C to 60 °C. Floxuridine release from DSPC/DSPG/Chol (70:20:10, mol/mol) liposomes at 40 °C and 45 °C was <10% over 20 min, however under these conditions, irinotecan encapsulation was <50% (data not shown). In contrast, >90% floxuridine release was observed within 5 min when the liposomes were incubated at ≥55 °C. The rapid drug release at this temperature occurred as a result of the liposomal membrane

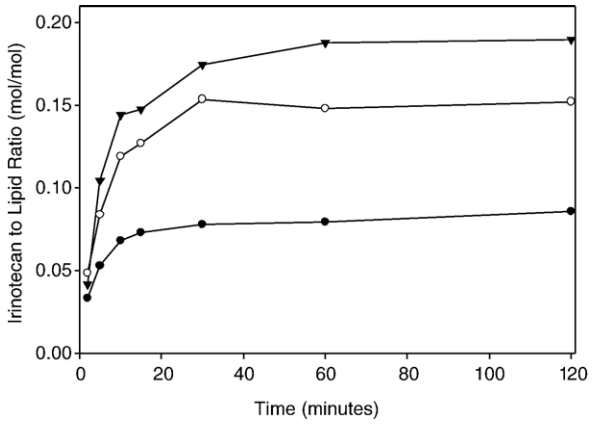

Fig. 6. The extent of irinotecan encapsulation at various starting drug to lipid ratios. Liposomes composed of DSPC/Chol/DSPG (70:10:20 molar ratio) were radiolabelled with [^{3}H]CHE and incubated with irinotecan at drug to lipid molar ratios of 0.1:1 (●), 0.2:1 (○) and 0.3 :1 (▼). The reaction was carried out at 50 °C for various times and assayed for drug encapsulation as previously described (representative data shown).

converting from a gel state to a liquid–crystalline state with a phase transition temperature (T_m) of 54.5 °C as measured using differential scanning calorimetry (data not shown). By decreasing the loading temperature to 50 °C, approximately 10% drug leakage was observed after 10 min, at which time irinotecan loading achieved maximum levels (see Fig. 3).

3.5. Simultaneous drug loading

In view of the difficulties associated with reproducibly achieving target irinotecan/floxuridine ratios inside DSPC/DSPG/Chol (70:20:10, mol/mol) liposomes with descending floxuridine entrapment due to leakage during irinotecan encapsulation, we examined alternative entrapment procedures whereby the two drugs could be simultaneously loaded subsequent to liposome formation and extrusion. We reasoned that if floxuridine could leak from liposomes when incubated at 50 °C, it should also be possible for the drug to load into preformed liposomes by following its concentration gradient and equilibrating across the liposome bilayer during irinotecan encapsulation. This approach relied on the ability of floxuridine and irinotecan to permeate the membrane under conditions where copper remained inside the liposomes. Liposomes were extruded in the presence of copper gluconate/TEA (pH 7.4) in the absence of floxuridine and then buffer exchanged to remove unencapsulated copper. Just prior to liposome loading, a single drug solution containing irinotecan and floxuridine was prepared. As shown in Fig. 8, floxuridine passively accumulated into liposomes simultaneously with irinotecan active encapsulation without compromising the integrity of irinotecan loading. The similar kinetics of liposome accumulation for floxuridine and irinotecan facilitated the ability to precisely control the drug/drug ratios in the final formulation and reliably achieve the target 1:1 molar ratio. It should be noted that the pharmacokinetics and drug release properties of this formulation did not differ from those observed with liposome formulations in which floxuridine and irinotecan were sequentially loaded (see Fig. 5).

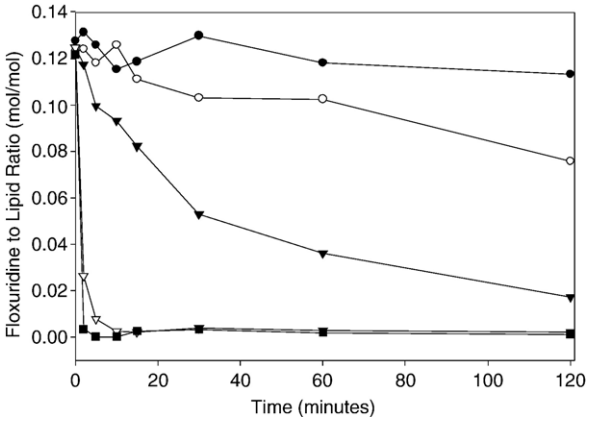

Fig. 7. The extent of floxuridine leakage from DSPC/Chol/DSPG (70:10:20 molar ratio) liposomes at various temperatures. Liposomes labelled with [^{14}C]CHE were extruded in the presence of 122 mM [^{3}H]floxuridine in HBS pH 7.4 and subsequently buffer exchanged to remove unencapsulated drug. To monitor the leakage rates of floxuridine from the formulation at a range of temperatures, the liposomes were incubated at 40 °C (●), 45 °C (○), 50 °C (▼), 55 °C (▽) and 60 °C (■) for 2 h. At various time points, aliquots were removed and passed though a Sephadex G-50 spin column and assayed for lipid and floxuridine content using liquid scintillation counting (representative data shown).

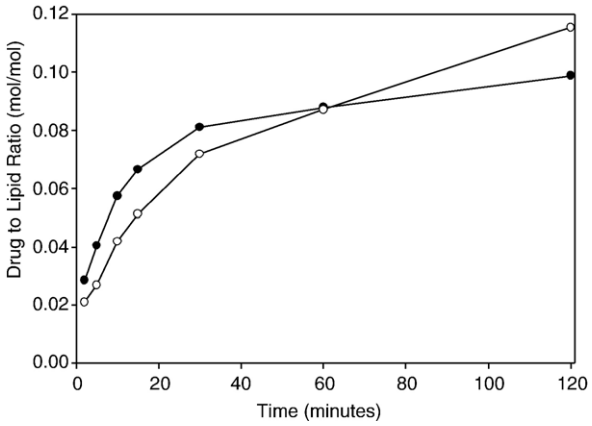

Fig. 8. The drug to lipid ratio of irinotecan and floxuridine during simultaneous drug loading. Liposomes composed of DSPC/Chol/DSPG (70:10:20 molar ratio) were radiolabelled with [^{14}C]CHE and incubated at 50 °C with [^{3}H] floxuridine and irinotecan as outlined in Section 2.4. At various time points aliquots were removed and passed through a Sephadex G-50 spin column to determine the irinotecan to lipid ratio (●) and the floxuridine to lipid ratio (○) (representative data shown).

4. Discussion

The use of drug combinations has been standard of care in the treatment of cancer for many years. Given this and the expanded use of liposomal delivery vehicles for cancer chemotherapy, it is somewhat surprising that very little research has focused on the development of liposomal drug combinations, either in separate liposomes or co-encapsulated in the same liposome. This may be related to expectations that the activity of liposomal drug combinations would simply be the sum of the individual liposomal drug components. In fact, the limited examples where liposomal drug combinations have been investigated resulted in therapeutic activity that was less than predicted for additivity [24,25]. Such adverse outcomes could have resulted from unfavorable pharmacological interactions such as restricted delivery of encapsulated drugs to tumor sites, counteractive drug activities or altered drug release rates.

We have recently identified a new and potentially important application for liposome delivery of drug combinations. This application arose from in vitro observations in cytotoxicity assays where the degree of drug synergy or antagonism has been shown to be dependent on the ratio of drugs in the combination [9]. The implications of this observation are that unless drug combinations are maintained in a synergistic range after administration in vivo, therapeutic activity will be compromised. Clearly, such control of systemic drug ratios after injection of unencapsulated drug cocktails is difficult, if not impossible, to achieve. Our approach to this problem is therefore to: (1) identify the optimum drug/drug ratio in a range of in vitro tumor cell lines, (2) design liposomes that can co-

encapsulate the desired drug/drug ratio and (3) maintain the drug/drug ratio in the synergistic range after administration so it can be exposed to tumor cells in vivo. One of the first drug combinations we applied this approach to was irinotecan and floxuridine, which are commonly used in the treatment of colorectal cancer [26–28]. This drug combination exhibited drug ratio-dependent synergy when examined in vitro where a 1:1 molar ratio was shown to be optimal over a broad range of tumor cell lines [9,29]. Consequently, we focused efforts to generate liposome formulations that could both encapsulate and maintain this drug combination at a 1:1 ratio.

In practical terms, the most challenging hurdle to achieving coordinated pharmacokinetics of drug combinations co-encapsulated in liposomes is the development of a single formulation that can simultaneously, yet independently control the release of two drugs that exhibit very differing physico-chemical properties, as is the case with irinotecan and floxuridine. Considering the significant differences in solubility properties of irinotecan and floxuridine, we contemplated establishing formulation conditions that would differentially control the entrapment and retention of these two drugs. As a very water-soluble compound (>400 mg/ml), floxuridine can be readily entrapped passively during liposome production and its release from liposomes will be predominately controlled by lipid permeability properties. In comparison, irinotecan can be actively entrapped inside liposomes with high efficiency using transmembrane pH gradients [30]. However, the use of pH gradients can be problematic due to the instability of phospholipids exposed to the acidic liposome interior which can lead to altered drug retention properties [31]. We therefore examined the use of encapsulated transition metals to actively load irinotecan without the use of pH gradients.

The rationale for utilizing encapsulated transition metals to actively load and retain irinotecan was based on the more than 40 years of evidence documenting interactions between transition metals and drugs [19]. We observed that only copper and zinc were able to promote the efficient encapsulation of irinotecan when high concentrations of liposome encapsulated unbuffered metal sulfates were utilized. The other metal sulfates tested (Ni, Mn, Co) provided little or no encapsulation under the conditions utilized even though $MnSO_4$ was previously reported to promote the encapsulation of doxorubicin into liposomes [32]. The pH of these unbuffered metal solutions ranged from a low of 3.3 for $MnSO_4$ to a high of 5.5 for $CoSO_4$. Based on this information and the loading curves, there appeared to be no correlation between the pH of the encapsulated salt solution and the extent of drug encapsulation. The lack of irinotecan uptake in the presence of $MnSO_4$ observed here contrasts the results obtained previously with another weak base camptothecin, topotecan, where liposomes containing this metal and the ionophore A23187 actively accumulated the drug [33]. This difference is related to the fact that in the presence of the ionophore, a transmembrane pH gradient (inside acidic) is generated which drives drug uptake. The fact that irinotecan does not load into the $MnSO_4$ without an ionophore indicates that the encapsulation observed here is not due to a pH gradient, but rather is related to selective interactions between irinotecan and the transition metals copper and zinc. The strength of the irinotecan interaction, correlates with the stability constants commonly observed with metal complexes [34]. Specific interactions between copper and the anthracyclines have been previously reported [35,36].

We were also able to achieve efficient loading using liposomes containing copper gluconate buffered to physiological pH with TEA, thereby alleviating potential difficulties with lipid degradation at acidic pH. When the influence of internal and external pH on the encapsulation of irinotecan was investigated, significantly lower irinotecan encapsulation was observed with decreasing pH. This may be the result of a decreased irinotecan permeability at lower pH due to a larger fraction of protonated amine, decreased interactions between irinotecan and copper gluconate/TEA or a combination of both. The mechanism responsible for this decrease has not been fully elucidated at this time. When the starting drug to lipid ratio was increased to 0.3:1, irinotecan encapsulation reached a maximum drug to lipid ratio of 0.18:1. This saturation effect suggests a stoichiometric relationship between irinotecan and copper gluconate/TEA. Further investigations are underway to fully characterize the nature of the interaction between irinotecan and copper gluconate/TEA inside liposomes. It is important to note that preliminary evidence for this buffer system indicates that TEA plays a major role in mediating the encapsulation and retention of irinotecan in contrast to the case of unbuffered copper sulfate where evidence for a direct irinotecan–copper interaction was obtained [37]. The copper gluconate/TEA irinotecan loading process described here provided an encapsulation system that was more amenable to pharmaceutical applications and was therefore utilized in an iterative optimization process to coordinate the release of irinotecan and floxuridine in vivo. This copper based formulation of irinotecan and floxuridine has been evaluated in dog toxicological studies and no evidence of copper toxicity was detected.

When floxuridine and irinotecan encapsulated in liposomes were administered i.v. to mice, surprising trends were observed in drug pharmacokinetics as a function of lipid composition. Historically, liposomes composed of saturated phospholipids and high cholesterol content (>33 mol%) have provided maximum drug retention in vivo for a wide range of drugs [38,39]. More recently, we demonstrated that for some drugs, low cholesterol-containing gel phase liposomes can reduce in vivo drug release compared to cholesterol-enriched systems [23]. In the studies here we observed a rather striking dependence of irinotecan and floxuridine leakage from DSPC-based liposomes in that the relationship between drug release and cholesterol content in the liposome was diametrically opposed for the two drugs. Whereas cholesterol-free DSPC/DSPG liposomes displayed optimal floxuridine retention, irinotecan was rapidly released from these systems. By titrating the cholesterol content in DSPC/DSPG liposomes containing floxuridine/irinotecan at a 1:1 ratio and assessing the plasma drug release properties after i.v. injection, we were able to identify that liposomes composed of DSPC/DSPG/Chol at a 70:20:10 molar ratio were able to coordinate the

pharmacokinetics of the two agents such that the circulating drug/drug ratio was maintained at approximately 1:1 for up to 24 h. The fact that the circulating drug to lipid ratios for both irinotecan and floxuridine decrease at the same rate over time indicates that both drugs are bioavailable and are being exposed systemically at the 1:1 ratio.

When liposomes were passively encapsulated with floxuridine and subsequently loaded with irinotecan, the temperature of loading had a dramatic impact on the drug content of the final formulation. In the cholesterol-free and low cholesterol formulations, irinotecan encapsulation was almost immediate at 60 °C. Unfortunately, this temperature resulted in over 90% floxuridine leakage within 2 min. This rapid leakage is likely the result of heating the liposomes above their transition temperature of 55 °C. Since these liposomes contain very little cholesterol, bilayer fluidity at these temperatures prevents adequate retention of floxuridine. Complete retention of floxuridine could be achieved at 40 °C, however, the encapsulation of irinotecan at this temperature was observed to be very poor. Consequently, a temperature of 50 °C was identified at which irinotecan could be encapsulated with high efficiency with manageable loss of floxuridine such that the target 1:1 drug ratio could be achieved. Although this was achievable in formulation batches less than 10 mL, it was not possible to achieve the desired accuracy and reproducibility in larger volumes.

In order to resolve this problem, an alternative loading scheme was developed based on the observed leakage of floxuridine at 50 °C. The high permeability of floxuridine near the phase transition temperature of the formulation allowed for the passive loading of floxuridine during irinotecan encapsulation. Analysis of the drug loading curves in Fig. 8 for the two drugs revealed very similar kinetics. This is somewhat surprising since the mechanisms driving the encapsulation of floxuridine and irinotecan are quite different and the two drugs display very different drug release dependence on cholesterol content. This method, however, has allowed us to control the encapsulated drug/drug molar ratio over a wide range of time. This unique dual loading method has the advantage over sequential loading in that the liposomes are prepared and extruded in the absence of chemotherapeutics and the final drug/drug ratio in the formulation can be tightly controlled.

This report describes one of the first attempts to co-formulate drug combinations in a manner that coordinates the pharmacokinetics of the different drugs after i.v. administration. By combining copper gluconate/TEA-based drug encapsulation and low-cholesterol containing gel phase liposomes, we were able to encapsulate and maintain irinotecan and floxuridine at a fixed, synergistic molar ratio of 1:1. We believe that maintaining this ratio will be critical to maximizing the therapeutic activity of this drug combination, based on results in tumor models [9,29]. As more cancer therapies utilize a greater number of agents, it will become increasingly important to avoid antagonistic drug interactions in order to maximize therapeutic activity. Since the pharmacokinetics of the individual drugs in conventional combination treatments cannot be controlled, formulating multiple drugs into delivery vehicles that can coordinate their pharmacokinetics is a viable approach to optimize the therapeutic activity of multiple agent combinations.

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