Liposomal Irinotecan: Formulation Development and Therapeutic Assessment in Murine Xenograft Models of Colorectal Cancer

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ABSTRACT

Purpose: **The purpose is to demonstrate whether an appropriately designed liposomal formulation of irinotecan is effective in treating mice with liver-localized colorectal carcinomas.**

Experimental Design: **Irinotecan was encapsulated in 1,2-distearoyl-*sn*-glycero-3-phosphocholine/cholesterol (55:45 molar ratio) liposomes using an ionophore (A23187)-generated transmembrane proton gradient. This formulation was evaluated *in vivo* by measuring plasma elimination of liposomal lipid and drug after i.v. administration. Therapeutic activity was determined in SCID/Rag-2M mice bearing s.c. LS180 tumors or orthotopic LS174T colorectal metastases.**

Results: **Drug elimination from the plasma was significantly reduced when irinotecan was administered in the liposomal formulation. At 1 hour after i.v. administration, circulating levels of the liposomal drug were 100-fold greater than that of irinotecan given at the same dose. High-performance liquid chromatographic analysis of plasma samples indicated that liposomal irinotecan was protected from inactivating hydrolysis to the carboxylate form. This formulation exhibited substantially improved therapeutic effects. For the LS180 solid tumor model, it was shown that after a single injection of liposomal irinotecan at 50 mg/kg, the time to progress to a 400-mg tumor was 34 days (as compared with 22 days for animals treated with free drug at an equivalent dose). In the model of colorectal liver metastases (LS174T), a median survival time of 79 days was observed after treatment with liposomal irinotecan (50 mg/kg, given every 4 days for a total of three doses). Saline and free drug treated mice survived for 34 and 53 days, respectively.**

Conclusions: **These results illustrate that liposomal encapsulation can substantially enhance the therapeutic activity of irinotecan and emphasize the potential for using liposomal irinotecan to treat liver metastases.**

INTRODUCTION

The liver is the primary site of blood borne metastases from cancers of several different origins, including carcinomas of the lung and breast. However, the liver is especially susceptible to metastases derived from colorectal adenocarcinomas. These metastases extravasate from the primary tumor site in the colon and access the liver directly via the portal circulation. Consequently, unlike secondary sites originating from gastric and pancreatic neoplasms, the liver is often the sole site of metastases for colorectal carcinoma. As many as 70% of patients with colorectal cancer will present with (synchronous) liver metastases at the time of their primary diagnosis or develop liver metastases (metachronous) as their disease progresses (1).

The management of liver-localized tumors is challenging. Despite overall 5-year survival rates of only 25 to 40%, surgical resection remains the treatment of choice for appropriately selected patients (2, 3). The most common therapeutic modality used in patients presenting with colorectal hepatic metastases is systemic chemotherapy. This palliative approach almost exclusively uses 5-fluorouracil–based treatment regimes, which yield responses in the range of 20 to 30%. However, most of these patients later develop resistance to these cytotoxic agents.

The emergence of irinotecan has had a major impact on chemotherapeutic regimens used to treat colorectal cancer. Irinotecan has demonstrated considerable therapeutic activity even in refractory disease. It is now combined with 5-fluorouracil and leucovorin for the treatment of advanced colorectal carcinoma. Irinotecan is a water-soluble analogue of the natural alkaloid camptothecin. Camptothecins act during the S-phase of DNA replication by stabilizing the complex formed between topoisomerase I and DNA, eventually resulting in lethal DNA breaks (4, 5). Topoisomerase I is overexpressed in several tumor types, including breast, lung, and colorectal tumors (6).

Liposomal carriers have the potential to improve the therapeutic activity of anticancer drugs. Improvements manifested by encapsulation of the active agent can include reduced toxicity, increased drug stability, improved drug distribution measures, and most importantly, improved therapeutic effects (7–9). Resultantly, liposomal formulations of doxorubicin (10–13) and daunorubicin (14, 15) have gained regulatory approval for clinical use. In addition to the licensed anthracyclines, other liposome-encapsulated cytotoxic agents are undergoing clinical assessment. Examples include various liposomal camptothecins in

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early-stage clinical trials (16, 17) and liposomal vincristine, demonstrating positive results in refractory patient populations (18, 19).

The advent of clinically available liposomal formulations of anticancer drugs is encouraging. Nevertheless, these achievements should be considered in the context of the anticipated value of liposomes as drug carriers. Encapsulation of numerous distinct therapeutic agents has failed to improve their therapeutic profile sufficiently in model systems to warrant clinical development. Others that have progressed to clinical trials have demonstrated limited use (20, 21). These observations may reflect the inherent limitations of the encapsulated drug. To date, the focus has been on liposomal formulations of approved cytotoxic drugs. When used as single agents, these drugs are not anticipated to elicit cures in the majority of clinical cancers (22). Furthermore, disease localization has the potential to minimize the effectiveness of liposomal therapy. These limitations are illustrated by the minimal activity associated with the majority of liposomal drug formulations designed for the treatment of liver-localized disease (23). This is disappointing given the natural tendency for liposomes to accumulate in the liver.

Liposomal uptake by the liver is a consequence of kupffer cell activity, fenestrated vascular beds, and the distinctive nature of hepatic circulation. The resultant distribution of the liposomes is considered, in conjunction with the choice of drug and the rate of drug release from the carrier, as one of the critical parameters that dictate the success of liposome-mediated treatment of liver neoplasms (24). The pattern and degree of liver accumulation can, to some extent, be controlled by manipulation of liposome composition and size (25).

This study describes efforts to improve the treatment of liver carcinomas by encapsulating irinotecan in liposomes. The unencapsulated drug has demonstrated considerable activity in the clinic despite the dependence of camptothecin-mediated cytotoxicity on maintenance of the closed lactone ring form of the drug. At physiologic pH, the closed lactone ring undergoes rapid hydrolysis to an inactive carboxyl or ring-opened form (reviewed in ref. 26). The ability of liposomes to maintain internal aqueous environments of low pH is an attractive means to stabilize the active lactone form of water-soluble camptothecins (27, 28).

Irinotecan is shown to be an ideal candidate for liposome encapsulation. An ionophore-mediated proton gradient was used to efficiently trap irinotecan in the acidic interior of the liposomes at relatively high drug-to-lipid ratios. Liposome encapsulation of irinotecan significantly increased the circulation longevity of the drug as compared with the free agent while maintaining the agent in its active lactone form. In comparison to free irinotecan, the liposomal formulation was significantly more effective in the treatment of two models of colorectal cancer.

MATERIALS AND METHODS

Materials. Irinotecan hydrochloride (Camptosar; Pharmacia (Upjohn, Mississauga, ON, Canada) was purchased from the British Columbia Cancer Agency Pharmacy. 1,2-disteroyl-*sn*-glycero-3-phosphocholine (DSPC) was purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol (Chol), the divalent cationic ionophore A23187, HEPES, and Sephadex G-50 (medium) were obtained from the Sigma Chemical Company (St. Louis, MO). Tritiated cholesteryl hexadecyl ether (NEN, Boston, MA) was used as a liposome marker, and [^{14}C]methylamine hydrochloride, used in determining liposomal pH gradients, was obtained from Amersham Biosciences Corp. (Baie d'Urfe, Quebec, Canada). 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI), used as a fluorescent lipid probe, was purchased from Molecular Probes (distributed by Cedarlane Laboratories Limited, Hornby, Ontario, Canada). All other chemicals used in this study were analytical or high-performance liquid chromatography (HPLC) grade. The LS180 and LS174T tumor cell lines were originally purchased from the American Type Culture Collection (Manassas, VA) and were maintained in tissue culture. Cells were used for experiments when they were between passages 3 and 20. After passage 20, the cells were discarded, and the new cell lines were brought up from frozen stock. Male SCID/Rag-2M mice (8 to 10 weeks old) were bred at the British Columbia Cancer Agency Joint Animal Facility.

Preparation of Liposomes. Large unilamellar vesicles were prepared according to previously published procedures (29). Briefly, the appropriate mixture of lipids were solubilized in chloroform and dried to a thin lipid film under a stream of N_2 gas, followed by incubation overnight under vacuum. The dried lipid films were subsequently hydrated in 300 mmol/L manganese sulfate (pH 3.4) to a final lipid concentration of 50 or 100 mg/mL. Hydration was enhanced by multiple freeze-thaw cycles (30) before 10 high-pressure passes through two-stacked polycarbonate membrane filters (100/80 nm pore size; Nuclepore, Pleasanton, CA) using an extruder (Northern Lipids, Vancouver, British Columbia, Canada) at 65°C. The size of the large unilamellar vesicles was typically in the range of 115 ± 15 nm based on quasi-elastic light scattering (Nicomp Particle Sizer model 270; NICOMP Particle Sizing Systems, Santa Barbara, CA). The external buffer was exchanged by running the sample down a Sephadex G-50 column equilibrated in 300 mmol/L sucrose, 20 mmol/L HEPES, and 15 mmol/L EDTA (SHE buffer) at pH 7.4. Liposomes composed of DSPC/Chol (55:45 mol%) or, if fluorescently labeled, DSPC/Chol/DiI (54.5:45:0.5 mol%). [^{3}H]CHE was incorporated into liposome formulations as nonexchangeable tracer (0.08 Ci/mol).

$MnSO_4$ pH Gradient Loading of Irinotecan. Irinotecan was actively loaded into DSPC/Chol (55:45 mol%) liposomes using an ionophore-mediated proton gradient (31). The divalent cation ionophore A23187 (0.5 μg per 1 mg lipid) was incorporated into the liposomal bilayer after incubation at 37°C or 60°C for 10 minutes. Subsequently, the efficiency of irinotecan encapsulation by liposomes (drug-to-lipid weight ratio of 0.3:1) was determined at incubation temperatures of 37°C or 60°C over a 2-hour period. During this process, the mixture was kept in the dark because irinotecan has been shown to undergo degradation upon extended exposure to light (32). Encapsulated drug was separated from free drug using a Sephadex G-50 mini spin column (33). Irinotecan was quantified by measuring absorbance at 370 nm using a Beckman DU-64 Spectrophotometer (Beckman Coulter Canada, Inc., Mississauga, Ontario, Canada). Lipid concentration was determined by liquid scintillation counting of [^{3}H]CHE (Packard 1900 scintillation counter; Packard Instrument Co., Inc., Meriden, CT).

The drug release characteristics of this formulation were assessed by dialyzing (12,000 to 14,000 MWCO, Fisher Scientific, Nepean, ON) the irinotecan-loaded liposomes against 4 liters of SHE buffer (pH 7.4) for 72 hours at 37°C. At 1, 2, 4, 24, 48, and 72 h, 3 × 50 μL aliquots were removed from the dialysis cassette and analyzed for encapsulated drug and lipid concentrations as described above.

Irinotecan Encapsulation as a Function of Transmembrane pH Gradient. Irinotecan was encapsulated by liposomes at drug-to-lipid weight ratios of 0.1, 0.2, 0.3, 0.4, and 0.5 using the $MnSO_4$ loading procedure. The loading efficiency was determined as described previously. The magnitude of the transmembrane pH gradients in the absence of drug or, at the drug-to-lipid weight ratios above, was assessed by incubating the samples at 23°C for 1 hour in the presence of [^{14}C]methylamine (34). Sephadex G-50 mini spin columns were used to separate encapsulated [^{14}C]methylamine from unencapsulated [^{14}C]-methylamine. The eluant and the original sample were assayed for [^{3}H] and [^{14}C] using a dual label analysis method. With the assumption that the liposomes used have a trapped volume of 1.2 μL/μmol lipid, the distribution of radioactive methylamine was used to calculate the magnitude of the transmembrane proton gradient.

Animal Husbandry. Male SCID/Rag-2M mice (8 to 10 weeks) were purchased from the Joint Animal Facility at the British Columbia Cancer Research Center. Mice were housed under standard conditions and had access to food and water *ad libitum*. The Committee on Animal Care, Research Ethics Office, Research Services at the University of British Columbia approved all protocols in accordance with the Guide to the Care and Use of Experimental Animals, Canadian Council on Animal Care. All animals were observed at least once a day, more if deemed necessary, during the pretreatment and treatment periods for mortality and morbidity. In particular, signs of ill health were based on body weight loss, change in appetite, behavioral changes such as altered gait, lethargy, and gross manifestations of stress. Animals were terminated when signs of severe toxicity or tumor-related illness be seen (CO_2 asphyxiation), and a necropsy was performed to assess other signs of toxicity.

Plasma Elimination Studies. Free irinotecan (50 mg/kg) and liposomal irinotecan (50 mg/kg irinotecan, 167 mg/kg lipid) were injected via the lateral tail vein into male SCID/Rag-2M mice. At the indicated time points, mice were terminated by CO_2 asphyxiation, and whole blood was collected by cardiac puncture and transferred to EDTA-coated tubes (Microtainers; Becton Dickinson, Franklin Lakes, NJ). Plasma was isolated by centrifuging the whole blood at 2500 × *g* for 10 minutes. The liposomal carrier present in the plasma was quantified by scintillation counting of the nonexchangeable and nonmetabolizable lipid marker [^{3}H]CHE. The concentration of total irinotecan and the relative percentages of the circulating drug existing as the lactone or carboxylate species were determined using HPLC analysis as described in ref. 35, with modifications. Briefly, irinotecan was extracted from plasma by diluting the sample in ice-cold methanol immediately before analysis. A standard curve for the lactone form of irinotecan was generated by dissolving the drug in DMSO. To generate an equivalent standard curve for the carboxylate species, irinotecan was processed in 50% MeCN: 50% 20 mmol/L borate buffer (pH 9). Separation was achieved at 35°C using a Symmetry C_{18} cartridge column (100Å, particle size, 5 μm; 250 × 4.6-mm inside diameter; Waters Corp., Milford, MA) with a Symmetry Sentry C_{18} guard column (particle size, 5 μm; 20 × 3.9 inside diameter; Waters Corp.) with a run time of 20 minutes at a flow rate of 1.5 mL/minute. A two-solvent mobile phase consisted of mobile phase A (75 mmol/L ammonium acetate, 7.5 mmol/L tetra-butylammoniumbromide, pH 6.4 adjusted with glacial acetic acid) and mobile phase B (100% acetonitrile), with the elution gradient containing a mixture of 76% A:24% B. For sample analysis, 80 μL of each sample and standard were loaded into 1-mL HPLC sample vials (Waters Corp.) with 200 μL inserts (Chromatographic Specialties, Inc., Brockville, Ontario, Canada), and 10 μL were injected into the column. Irinotecan was quantified using a HPLC system equipped with a Waters Model 717 plus autosampler (set to 4°C), a Model 600E pump and controller, and a Model 470 Scanning Fluorescence Detector (Waters Corp.) set at an excitation wavelength of 362 nm and an emission wavelength of 425 nm. Data were acquired and processed with the Millenium32 chromatography manager (version 3.20; Waters Corp.).

Establishing a Maximum-tolerated Drug Dose. Limited dose ranging studies were used in an effort to determine the maximum-tolerated dose of free and liposomal irinotecan. Male SCID/Rag-2M mice in groups of two were given free or encapsulated drug by single or multiple [given every 4 days for a total of three doses (3 × q4d)] i.v. injections into the lateral tail vein. Qualified animal care technicians monitored the mice for weight loss and other signs of stress/toxicity for a period of 30 days. Individual animals that lost >30% of their original weight or those appearing severely stressed, as judged by appearance and/or behavior, were terminated. After 30 days, all remaining animals were terminated by CO_2 asphyxiation, and necropsies were conducted to identify any additional drug toxicities. Toxicity studies aimed at determining the exact LD_{10} dose of a drug formulation are neither sanctioned by the Canadian Council on Animal Care nor by the Committee on Animal Care, Research Ethics Office, Research Services at the University of British Columbia.

Liposomal Irinotecan Antitumor Efficacy Using the Human LS180 Solid Tumor Model. Male SCID/Rag-2M mice (20 to 30 g, six per group) were inoculated with 1 × 10^6 LS180 cells s.c. on the right posterior dorsal side. Once the tumor had established and reached a size accurately measurable with calipers (tumor volume, >0.04 cm^3; day 11 postinoculation), free or encapsulated irinotecan dosing schedules of single or multiple (days 11, 15, or 19) injections of 50 or 100 mg/kg irinotecan via the lateral tail vein were initiated. Animals were monitored daily for any signs of stress or toxicity and were terminated when animals displayed increasing health deterioration including weight loss, lethargy, scruffy coats, or dehydration. Survival time for terminated animals was recorded as the following day. Animals were monitored for 60 days or until all animals had been terminated. Drug-mediated delays in tumor growth were estimated by extrapolation of a line tangential to exponential tumor growth down to the *X* axis, which indicated a time in days post cell inoculation.

Liposomal Irinotecan Antitumor Efficacy Using the Human LS174T Orthotopic Tumor Model. Male SCID/Rag-2M mice (20 to 30 g, six per group) were inoculated intrasplenically using the method developed by Kozlowski *et al.* (36–39). Briefly, a small incision was made in the skin lateral to the midline of the mouse. An additional incision (2 to 4 mm) in the abdominal wall allows exteriorization of the spleen. Fifty microliters (5×10^6 cells) of LS174T cell suspension were injected just under the capsule of the spleen. After inoculation, the spleen was returned to the abdominal cavity and the incision sutured. Wound clips were used to close the skin. Histologic assessment of the liver after intrasplenic inoculation indicated that at day 7, LS174T cells were well established in the liver. This reflects the previously reported observations (36). On day 7 after cell inoculation, animals began q4d dosing schedules for three injections (days 7, 11, or 15) of 50 mg/kg free or liposomal irinotecan given via the lateral tail vein. Animals were monitored daily and terminated upon signs of severe weight loss (>30%) and/or health deterioration as above. Survival time for terminated animals was recorded as the following day.

Liposome-mediated Drug Delivery to LS174T Liver Metastases. To illustrate liposome delivery to tumors in the liver, male SCID/Rag-2M mice (20 to 30 g, two per group) were inoculated intrasplenically as described in the previous section. Once the tumors were well established in the liver (day 19 after inoculation), animals were given a single dose of liposomal irinotecan (50 mg/kg) or mock-loaded DSPC/Chol/DiI (54.5:45:0.5 mol%) liposomes injected via the lateral tail vein. Twenty-four hours later, animals were terminated by CO_2 asphyxiation, whole blood was collected via cardiac puncture as outlined in the plasma elimination studies, and a portion of a lobe of the liver was harvested for sectioning and histochemistry. DiI inclusion in the liposomes enabled their visualization using fluorescent microscopy (λ_{excit}, 549 nm; λ_{emit}, 565 nm). Liver sections taken from untreated animals ($n = 2$) were H&E stained to reveal tumor localization.

Statistical Analysis. One-way ANOVA was performed on the tumor volume results obtained after the administration of free or liposomal irinotecan. Common time points were compared using the post hoc comparison of means (Scheffé test). Survival data were analyzed using a multiple-sample test in the Survival Analysis module of Statistica (Statsoft, Inc., Tulsa, OK). This test is an extension of Gehan's generalized Wilcoxon test, Peto and Peto's generalized Wilcoxon test, and the log-rank test. Differences were considered significant at $P < 0.05$.

RESULTS

Irinotecan Encapsulation by DSPC/Chol (55:45 mol%) Liposomes. The incorporation of an ionophore into the lipid bilayer can generate a stable transmembrane pH gradient. The ionophore exchanges cations present in the internal buffer of the liposomes for protons from the external buffer, thereby generating and maintaining a pH gradient. In this study, the divalent cation ionophore, A23187, facilitates the outward movement of Mn^{2+} ions across the bilayer in exchange for the inward movement of $2H^+$.

Fig. 1 illustrates the importance of the A23187 ionophore in mediating efficient encapsulation of irinotecan by liposomes composed of DSPC and Chol (55:45mol%). In the absence of A23187, drug accumulation in the liposomes was <20%, irrespective of the incubation temperature used. In the presence of A23187, incubation at 60°C resulted in >98% of the added irinotecan being incorporated into the liposomes after 15 minutes (Fig. 1*A*). In contrast, <30% of the irinotecan was associated with the liposomes at the end of 2 hours of incubation at 37°C (Fig. 1*B*).

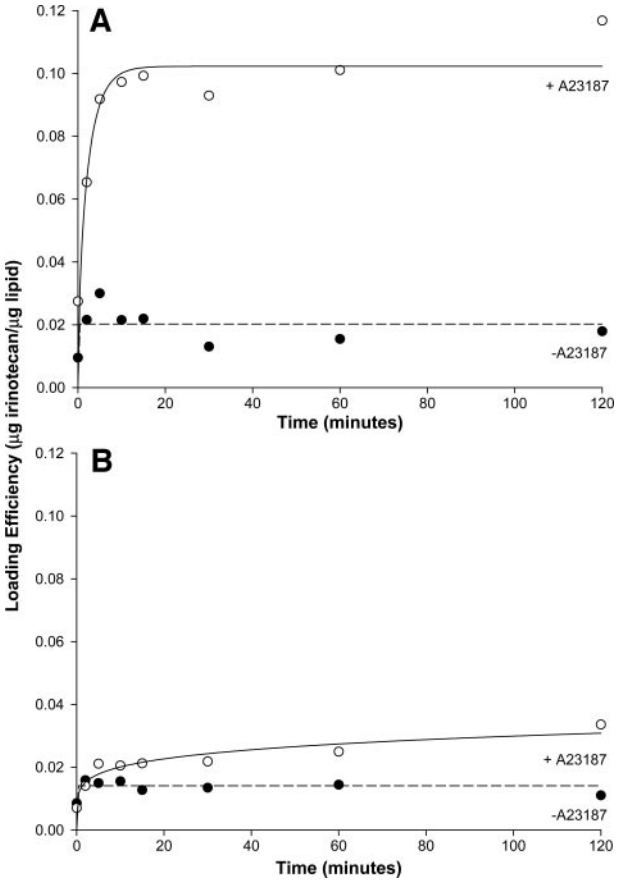

Fig. 1 Effect of the A23187 ionophore on pH gradient-mediated loading of irinotecan into DSPC/cholesterol (55:45 mol%) liposomes at 60°C (*A*) or 37°C (*B*). Loading was evaluated at two temperatures in the presence (○) or absence (●) of the A23187 ionophore at an initial drug-to-lipid ratio of 0.1:1 (wt:wt). Drug encapsulation was determined as described in Materials and Methods.

To determine the maximum level of irinotecan encapsulation achievable using this loading method, the starting drug-to-lipid weight ratio was increased incrementally from 0.1 to 0.5. The results, shown in Fig. 2*A*, indicate that the loading efficiency at 60°C decreases as the added drug-to-lipid weight ratio increases. Loading efficiencies of >80% were achieved for samples incubated for 1 hour at starting drug-to-lipid weight ratios of 0.1 to 0.4. When the starting drug-to-lipid weight ratio was 0.5, the loading efficiency was <70%. Because drug loading is anticipated to be dependent on the magnitude and stability of the ionophore-generated pH gradient, the transmembrane pH gradients of the samples were determined after 1 hour of incubation at 60°C (Fig. 2*B*). In the presence of A23187, but in the

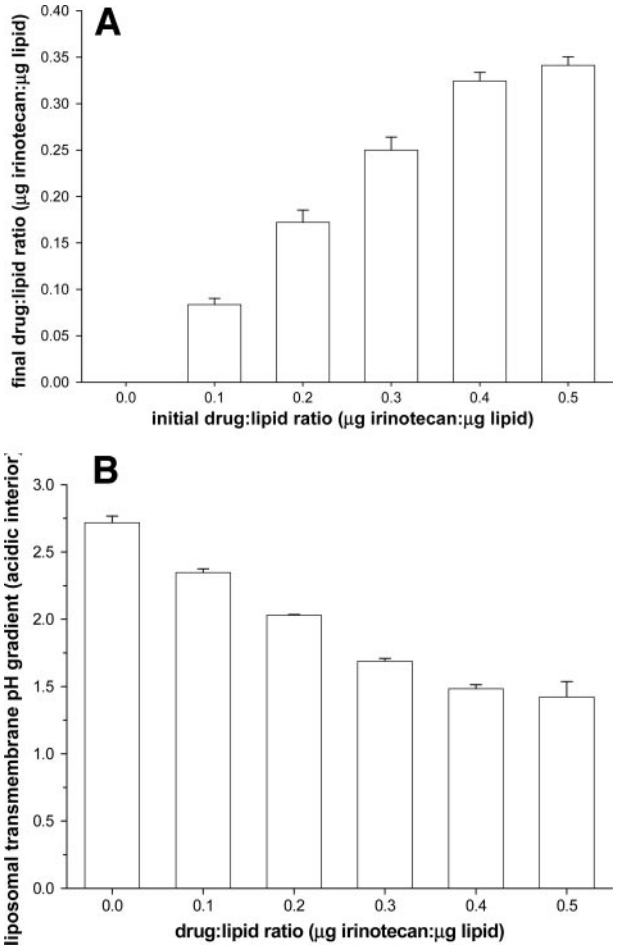

Fig. 2 The effect of the initial drug-to-lipid weight ratio on irinotecan loading (*A*) and transmembrane pH gradient before and after loading (*B*). *A.* DSPC/cholesterol liposomes (55:45 mol%) were incubated at 60°C for 1 hour at different drug-to-lipid weight ratios, and the extent of irinotecan encapsulation was determined (see Materials and Methods) and expressed as the average final drug-to-lipid weight ratio ± SD. *B.* Transmembrane pH gradients were determined as described in Materials and Methods using [^{14}C]methylamine as a probe. The [^{14}C]methylamine was added after 1 hour of incubation at 60°C in the absence of added drug or in the presence of the indicated irinotecan-to-lipid weight ratio.

absence of added drug, a transmembrane pH gradient of approximately three units was detected using the pH probe methylamine. The magnitude of this pH gradient decreased to 1.5 units when the initial drug-to-lipid weight ratio was ≥0.4. Therefore, reduced loading efficiency could be attributable to lower transmembrane pH gradients, but regardless of the loading efficiency, all formulations exhibited a significant transmembrane pH gradient (inside acidic) after loading. The loading efficiency at all drug-to-lipid weight ratios tested was not improved by increasing the incubation time period beyond 1 hour. Indeed, the majority of irinotecan encapsulation occurred during the first 30 minutes of incubation (data not shown).

From the data presented thus far, it has been demonstrated that irinotecan can be efficiently loaded into DSPC/Chol liposomes at an initial drug-to-lipid weight ratio of 0.3. Moreover, the resultant drug-loaded liposomes were able to retain a transmembrane pH gradient of >1.5 units. On the basis of these observations, the stability of this formulation was initially assessed by dialyzing the sample at 37°C against a large volume of buffer (pH 7.4). After 72 hours of incubation, <2% of the encapsulated irinotecan was released (data not shown). These results indicated that this formulation was worthy of additional evaluation.

***In vivo* Plasma Elimination of Free and Liposomal Irinotecan.** The plasma elimination profiles of both free irinotecan (50 mg/kg) and irinotecan encapsulated in DSPC/Chol liposomes (50 mg/kg drug, 167 mg/kg lipid) were determined after i.v. injection into SCID/Rag-2M mice. At 1, 4, and 24 hours after injection of the liposomal formulations or 5, 15, and 60 minutes after injection of free irinotecan, plasma was collected and analyzed for drug and/or liposomal lipid levels (Fig. 3).

DSPC/Chol liposomes exhibited an extended circulation life span and the elimination of irinotecan-loaded (Fig. 3*A*, filled symbols) and mock-loaded (Fig. 3*A*, open symbols) liposomes was not significantly different. Approximately 40% of the injected lipid dose could still be found in the plasma compartment 24 hours after i.v. injection. It should be noted that these circulation levels were achieved in the absence of surface grafted polyethylene glycol and are reasonably high because of the high lipid dose used (167 mg/kg).

Free irinotecan was eliminated rapidly from the plasma compartment (Fig. 3*B*; Table 1). In contrast, liposome-encapsulated irinotecan was maintained at higher irinotecan levels throughout the 24-hour time frame of the experiment. At 1 hour, the measured levels of liposomal irinotecan in the plasma were 100 times greater than those associated with the free drug (Fig. 3 and Table 1).

The therapeutic activity of irinotecan is dependent on maintenance of the drug in its closed lactone ring form. This is of particular importance when considered in the context of physiologic pH, which favors the existence of the inactive open-ring carboxyl form of irinotecan (40). Resultantly, the relative proportions of the lactone and carboxyl forms of irinotecan responsible for the plasma levels of total irinotecan presented in Fig. 3*B* were quantified by HPLC analysis. The results (summarized in Table 1) suggest that at 5 minutes after injection of the free drug, ~28% of the measured irinotecan was in the carboxyl form, and this increased close to 60% at the 1-hour time point. In contrast, >95% of the measured liposomal irinotecan was in the lactone form at 1 and 4 hours. This indicates that irinotecan remains encapsulated in the low pH environment of the liposomes, thereby maintaining its lactone configuration. At 24 hours after injection of the liposomal drug, the irinotecan concentration in plasma was 100.90 μg/mL or ~8% of that measured at the 1-hour time point (1263.63 μg/mL; see Table 1). At this time point, >80% of the remaining drug exists in the lactone form and suggests that the vast majority of the detected drug is likely present in circulating liposomes that exhibit a low interior pH.

By calculating the ratio of irinotecan to liposomal lipid from the data shown in Fig. 3, *A* and *B*, and Table 1, it is possible to illustrate the loss of drug from liposomes after i.v. injection. This estimate assumes that 100% of the measured irinotecan in the plasma compartment is associated with circu-

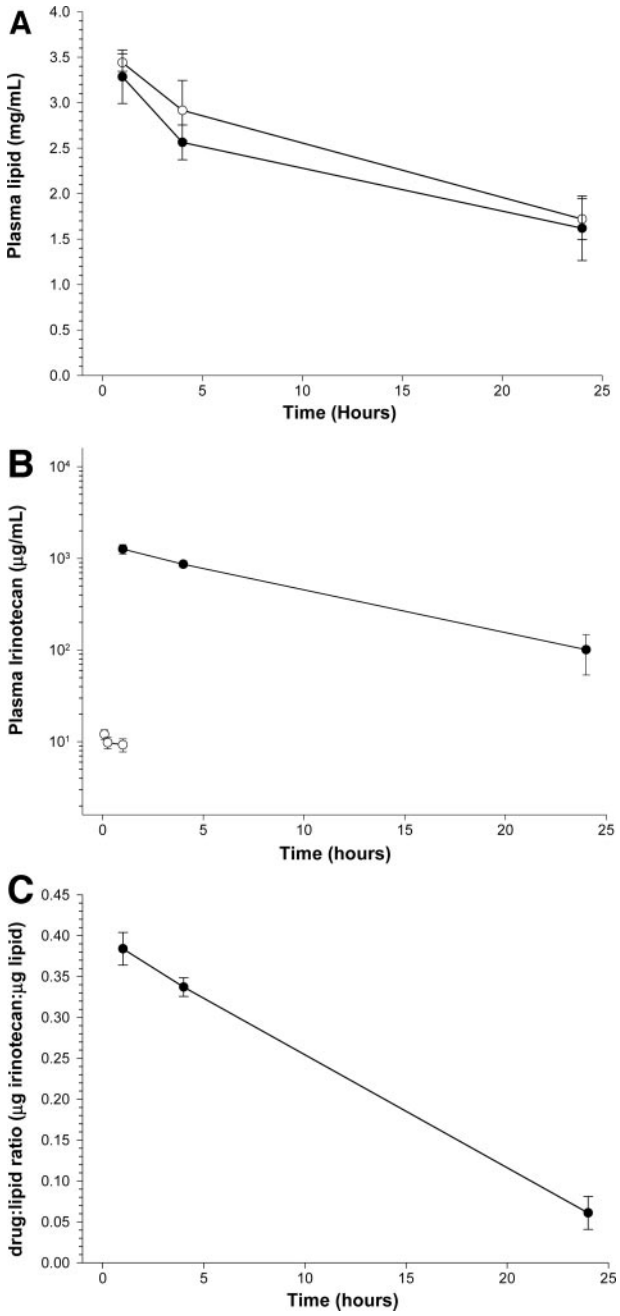

Fig. 3 Plasma levels of free and liposomal irinotecan determined at three time points after i.v. administration in SCID/Rag-2M mice. *A*. Mice received injections of liposomal irinotecan (drug-to-lipid weight ratio of 0.3) (●) or mock-loaded liposomes (○) at a lipid dose of 167 mg/kg and a drug dose of 50 mg/kg. At the indicated times, plasma was collected and the circulating liposomal lipid concentration was determined as described in Materials and Methods. *B*. Plasma levels of total irinotecan (carboxyl form + lactone form) after i.v. injection of the liposomal drug formulation (●) or free drug (○) at a dose of 50 mg/kg drug. *C*. Using the measured data shown in *A* and *B*, changes in drug-to-lipid weight ratio can be calculated at the indicated time points. Decreases in the drug-to-lipid ratio provide a measure of drug release from liposomes in the plasma compartment. All *data points* represent the mean values obtained from six mice per time point, and the *error bars* represent the SD.

lating liposomes. As shown in Fig. 3*C*, irinotecan is released at a steady rate from the liposomes over the 24-hour time course.

The Efficacy of Liposomal and Free Irinotecan in Treating an Established LS180 Human Xenograft Tumor Model. Efficacy studies were undertaken to determine whether liposome encapsulation engendered improvements in the activity of the irinotecan when compared with the free drug. The therapeutic benefits of the free and encapsulated drug were assessed in two advanced colorectal tumor models.

The Canadian Council of Animal Care does not authorize formal toxicity studies. Therefore, the maximum-tolerated dose of free and liposomal irinotecan was determined through limited dose range finding studies in tumor-free SCID/Rag-2M mice (two mice per dose). Unfortunately, due to the nature of commercially available irinotecan [20 mg/mL irinotecan dissolved in 45 mg/mL sorbitol, 0.9 mg/mL lactic acid (pH 5)], the dose escalation studies with free drug were inconclusive. A pronounced acute dose-limiting toxicity was observed when the drug was given at doses $>$ 100 mg/kg. This toxicity was observed within 1 minute of injection, suggesting that it was a consequence of the formulation as opposed to drug-induced effects on normal cell populations typically observed as weight loss and/or myelosuppression. An evaluation of drug-induced weight loss during the dose escalation studies with DSPC/Chol irinotecan (data not shown) indicated that the drug was tolerated (on injection) at the 100 mg/kg dose but was toxic as judged by weight loss. Necropsies of the injected animals 30 days after drug administration revealed no gross abnormalities in any of the tissues examined. On the basis of these limited toxicity studies, efficacy experiments focused on drug doses of 50 and 100 mg/kg, given as a single injection or 3 $\times$ q4d.

Studies completed in LS180 tumor-bearing SCID/Rag-2M mice evaluated drug-induced weight loss as a measure of toxicity (Fig. 4) and drug-induced delays in tumor growth as a measure of efficacy (Fig. 5). Measurable LS180 solid tumors were obtained 11 days after s.c. inoculation of 10^6 LS180 cells, and treatment was initiated at this time using a single dose or a 3 $\times$ q4d schedule.

Weight loss results clearly indicate that the liposomal irinotecan formulation was more toxic than the free drug when administered three times at 100 mg/kg on the q4d schedule (Fig. 4*D*). The nadir in weight loss after this treatment schedule occurred between 2 and 7 days after the final drug dose. At this time, animals treated with liposomal drug had lost $>$30% of their original body weight and had to be terminated. It should be noted that other indicators of toxicity were not evident, and the animals were terminated solely because of weight loss considerations at this time. In general, animal care protocols indicate that animals must be terminated when body weigh loss is $>$20%; however, protocols used in these studies emphasized a number of different end points, and if animals were considered healthy as judged by other end points, then animals with $>$20% body weigh loss were maintained on the study. Animals treated with 50 or 100 mg/kg free irinotecan or liposomal irinotecan at a dose of 50 mg/kg exhibited a weight loss in the range of 7 to 13%, suggesting that these drug doses were reasonably well tolerated (Fig. 4*A*–*C*).

Results obtained after treatment of mice bearing established LS180 tumors have been summarized in Fig. 5. These

Table 1 HPLC analysis of plasma irinotecan after i.v. administration of free or liposomal drug

Time	Plasma concentration (μg/mL)			Irinotecan (% of total)	
	Lactone	Carboxylate	Total	Lactone	Carboxylate
Free irinotecan					
5 minutes	8.72 ± 0.93	3.34 ± 0.58	12.06 ± 1.42	72.45 ± 2.38	27.55 ± 2.38
15 minutes	5.44 ± 0.79	4.35 ± 0.80	9.79 ± 1.43	55.67 ± 3.37	44.33 ± 3.37
1 hour	3.80 ± 0.61	5.46 ± 0.96	9.26 ± 1.51	44.12 ± 6.52	55.85 ± 6.52
Liposomal irinotecan					
1 hour	1236.66 ± 148.67	26.97 ± 5.84	1263.63 ± 151.43	97.87 ± 0.41	2.13 ± 0.41
4 hour	838.89 ± 66.41	25.46 ± 5.00	864.35 ± 66.35	97.02 ± 0.62	2.98 ± 0.62
24 hour	82.56 ± 46.10	18.34 ± 9.36	100.90 ± 47.47	79.32 ± 8.68	20.68 ± 8.68

NOTE. Plasma levels of irinotecan were determined as described in Materials and Methods. $n = 6$, values quoted are means ± SD.

data clearly establish that the DSPC/Chol irinotecan formulation was therapeutically more active than free drug. The tumors of untreated animals progressed to a size of 400 mg 19 days after inoculation (Fig. 5*A*). A single dose of 50 mg/kg free irinotecan delayed this progression to day 22. Increasing the dose of free irinotecan to 100 mg/kg slowed the rate of tumor growth further, such that a size of 400 mg was recorded on day 26 (Fig. 5*A*). The equivalent values observed for the liposomal formulations are shown in Fig. 5*B*. A single dose of 50 mg/kg liposomal irinotecan prevented the tumor mass reaching 400 mg until day 34. This growth retardation was extended to 39 days after one dose of 100 mg/kg liposomal irinotecan.

Additional gains in efficacy were observed for both free and liposomal irinotecan when the administration schedule was increased to three doses administered on days 11, 15, and 19 (Fig. 5, *C* and *D*). Tumors reached 400 mg in size on day 30 after three doses of 50 mg/kg free irinotecan (Fig. 5*C*). The tumor growth curve representing three doses of free 100 mg/kg irinotecan resembles that observed after a single dose of 100 mg/kg liposomal irinotecan, with tumors reaching 400 mg after 40 days

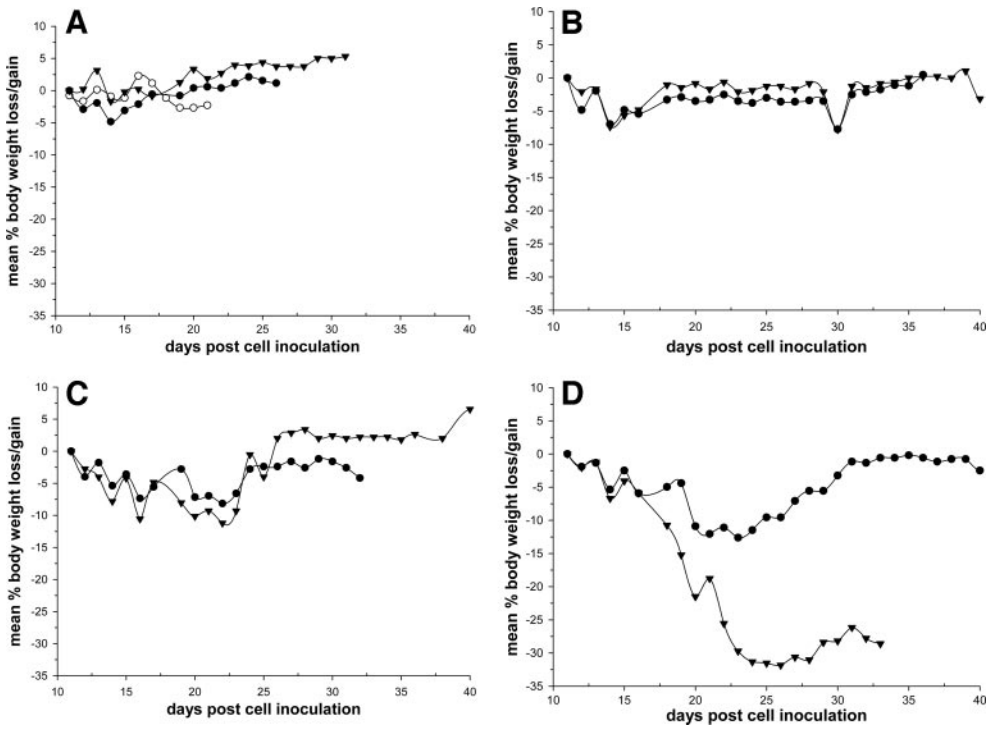

Fig. 4 Toxicity of free (*A* and *C*) and liposomal (*B* and *D*) irinotecan as assessed by weight loss in LS180 tumor-bearing mice. Single injections (*A*) free irinotecan or (*B*) liposomal irinotecan (drug-to-lipid weight ratio 0.3) were administered i.v. on day 11 after cell inoculation (1×10^6 cells/50 μL), or three injections of (*C*) free irinotecan or (*D*) liposomal irinotecan (drug-to-lipid weight ratio 0.3) were administered on day 11, 15, and 19 after cell inoculation. Changes in weight were obtained from saline-treated animals (○); animals given 50 mg/kg irinotecan/dose per injection (●); and animals given a 100 mg/kg irinotecan/dose per injection (▼). For clarity purposes, only the mean change in weight (relative to the weights of animals determined on day 10) is shown on the indicated days. Saline-treated animals were terminated on day 23 because of progression and occasional ulceration of the solid tumors. Mice that received three 100 mg/kg injections of liposomal irinotecan were terminated on day 34 because of drug-induced toxicity manifested as severe weight loss. However, it should be noted that these animals appeared healthy by all other measurements of stress.

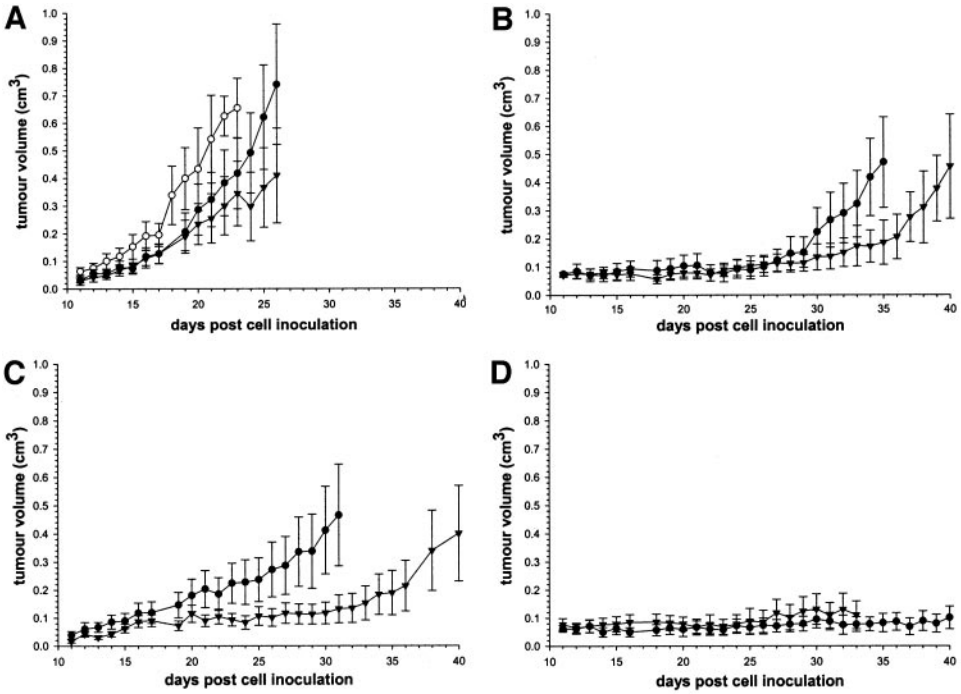

Fig. 5 Antitumor efficacy of free (*A* and *C*) and liposomal (*B* and *D*) irinotecan as determined in LS180 tumor-bearing animals. Drug efficacy was assessed by measuring tumor volume as a function of time after s.c. injection of LS180 tumor cells (1×10^6 cells/50 μL, day 0). Single injections of (*A*) free irinotecan or (*B*) liposomal irinotecan (drug-to-lipid weight ratio 0.3) were administered i.v. on day 11, or three injections of (*C*) free irinotecan or (*D*) liposomal irinotecan (drug-to-lipid weight ratio 0.3) were given on days 11, 15, and 19. The data shown represents the mean and SE derived from a group size of six mice. These data were obtained from saline-treated animals (○); animals given a 50 mg/kg irinotecan/dose per injection (●); and animals given a 100 mg/kg irinotecan/dose per injection (▼).

(compare Fig. 5*C* with Fig. 5*B*). Administration of three doses of 50 mg/kg or 100 mg/kg liposomal irinotecan mediated substantial delays in onset of tumor growth. There were no increases in tumor size recorded over the 40-day evaluation period (Fig. 5*D*).

Efficacy of Single and Multiple Dose Administration of Liposomal and Free Irinotecan in the Orthotopic LS174T Human Tumor Model. The results shown in Fig. 5 suggest that the liposomal formulation of irinotecan is therapeutically more active than free drug, but these results were achieved using a s.c. tumor model. The following studies were initiated to determine whether similar gains in therapeutic activity could be achieved using a murine LS174T model designed to mimic liver metastases arising from colorectal cancer (39). The LS174T is a relatively slow-growing model, and the effects of a large tumor burden do not become apparent until approximately day 30 after inoculation, after which, the host condition deteriorates quickly, and the animals must be terminated (Fig. 6). Thus, in the absence of treatment (saline control), the median survival time of inoculated animals was determined to be 34 days. Animals treated with free irinotecan ($3 \times$ q4d) exhibited a median survival of 53 days, whereas those treated using the same schedule with liposomal drug exhibited a median survival time of 79 days. This represented a 141% increase in life span, which was significantly greater ($P < 0.05$) than the 56% increase in life span achieved using an identical dose of free irinotecan. It should be noted that treatment was initiated 7 days after tumor inoculation. Studies with control mice terminated 7 days after LS174T cell injection indicated the presence of established disease in the liver (results not shown).

Liposome-mediated Delivery to Liver Metastases. To determine whether improved therapy was associated with liposome delivery to regions of LS174T tumor growth within the liver, LS174T tumor-bearing animals were dosed with a liposomal irinotecan formulation prepared with 0.5 mol% DiI, a nonexchangeable fluorescent marker (41). Representative photomicrographs are shown in Fig. 7.

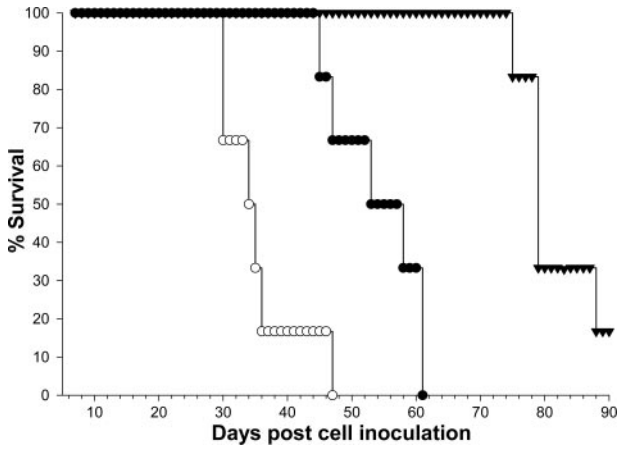

Fig. 6 Antitumor efficacy of free and liposomal irinotecan as determined in the orthotopic LS174T tumor model. Mouse survival was determined as a function of time after intrasplenic injection of LS174T tumor cells (5×10^6 cells/50 μL, day 0) as described in Materials and Methods. Survival cannot be used as an experimental end point. Therefore, multiple indicators of animal stress were used to assess tumor progression. If an animal was terminated because of tumor progress, the day of death was recorded as the subsequent day. The technician assessing animal stress was blinded to the study groups. Three injections of 50 mg/kg free or liposomal drug (drug-to-lipid weight ratio 0.3) were administered i.v. on days 7, 11, and 15 after cell inoculation ($n = 6$). The data shown were obtained from saline-treated animals (○); animals given free irinotecan (●); and animals given liposomal irinotecan (▼).

The tumor-bearing livers of untreated animals are shown in Fig. 7*A*. This image is characterized by the presence of large vacuole-like structures observed within regions of tumor growth. Because the LS174T tumors are derived from a mucin-producing adenocarcinoma cell line (42), it can be suggested that these structures may be mucin-containing vacuoles. Both empty (Fig. 7*B*) and irinotecan-loaded (Fig. 7*C*) liposomes were seen predominantly in regions of healthy liver tissue (thin arrows), as opposed to regions of tumor growth (large arrows). These images suggest that DSPC/Chol liposomes accumulate at the periphery of the tumor with no evidence of tumor localization 24 hours after i.v. administration.

DISCUSSION

DSPC/Chol (55:45 mol%) liposomes (~100 nm in diameter) proved to be an effective carrier for irinotecan. Encapsulation of irinotecan was mediated by the combination of 300 mmol/L $MnSO_4$ solution in the interior of the liposomes and A23187 incorporated into the liposomal bilayer. A23187 is capable of transferring two protons into the liposome for every one Mn^{2+} ion transported out. This creates a pH gradient across the liposomal membrane, which was crucial for efficient loading (>90%) of irinotecan (Fig. 1). Under these conditions, high drug-to-lipid weight ratios of 0.3 could be achieved (Fig. 2). The formulation proved to be stable after incubation in buffer at 37°C for 72 hours and therefore was considered a suitable candidate for further investigation.

The plasma elimination studies illustrated in Fig. 3, demonstrated that the encapsulated irinotecan was steadily released from the liposomes over a 24-hour period. Approximately 70% of the initial dose of liposomal irinotecan was present at 4 hours postinjection. This decreases to <10% after 24 hours. HPLC analysis revealed that the circulating drug associated with the liposomes was maintained in the active lactone form (Table 1). This is a consequence of irinotecan encapsulation within the favorable acidic environment of the liposomes. These observations are in contrast to the data recorded for free irinotecan. Very low concentrations of free drug were recorded in the plasma compartment 5 minutes postinjection. At the 15-minute and 1-hour time points, there was no marked change in total drug levels; however, there was a progression from the lactone form toward the inactive carboxyl form, as would be expected under physiologic conditions. The rapid disappearance of irinotecan from the circulation probably reflects the high concentrations of esterases present in mouse plasma. Resultantly, irinotecan is rapidly converted into its more active congener, SN-38 (43). SN-38 would not be detectable under the HPLC conditions used here.

Efficacy studies conducted in SCID/Rag-2M mice bearing established s.c. LS180 solid tumors indicated that free irinotecan was effective in slowing tumor growth. However, this effect was much more pronounced after administration of the liposomal drug, with tumor progression arrested over an extended time period (Fig. 5). These observations were extended to mice inoculated intrasplenically with LS174T cells. This orthotopic tumor model is designed to mimic liver metastases secondary to colorectal cancer (39). Liposomal irinotecan demonstrated significantly greater survival ($P < 0.05$) than free drug at equivalent doses in this model (Fig. 6).

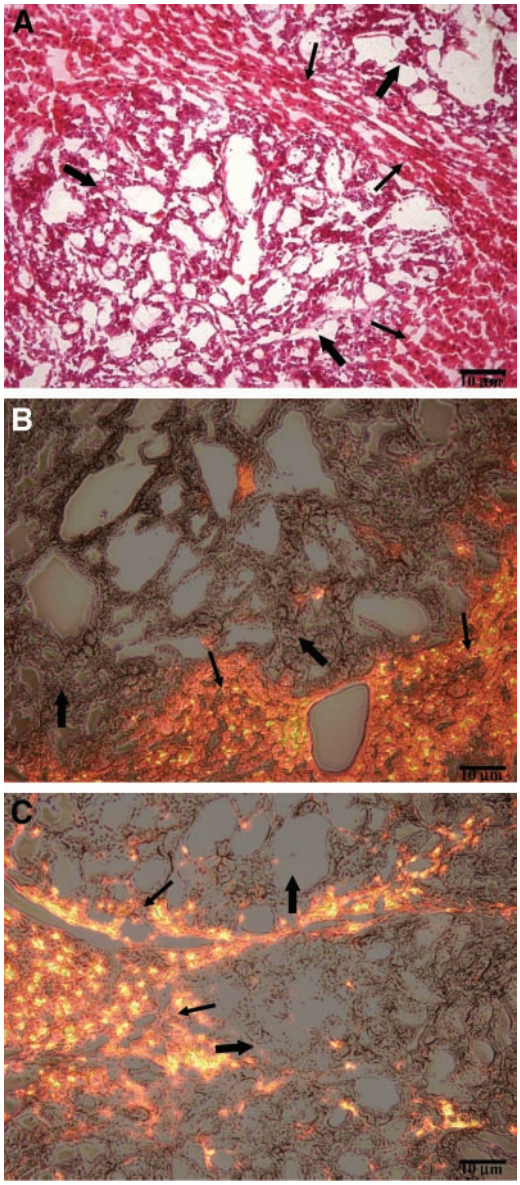

Fig. 7 Liposome-mediated drug delivery to livers of LS174T tumor-bearing mice. DiI-labeled DSPC/Chol liposomes, empty or irinotecan-loaded (drug-to-lipid weight ratio 0.3), were injected i.v. into mice (two per group) inoculated with LS174T tumor cells 19 days earlier as described in Materials and Methods (5×10^6 cells/50 μL). Twenty-four hours after injection of saline (*A*), mock-loaded liposomes (*B*) and irinotecan-loaded liposomes (*C*), the livers were harvested for histology as indicated in Materials and Methods. *A*. Representative H&E staining where the (*thick arrows*) indicate regions of tumor growth, whereas the (*narrow arrows*) indicate normal liver tissue; *B*, representative fluorescent micrograph of liver section derived from a mouse injected with a mock loaded liposome (no encapsulated drug); and *C*, representative fluorescent micrograph of liver section derived from a mouse injected with irinotecan-containing liposomes.

The results described clearly demonstrate that liposomal encapsulation can potentiate the therapeutic activity of irinote-

can against models of human colorectal cancer and associated liver-localized metastases. This supports our belief that liposomal anticancer formulations encapsulating appropriately selected drugs can be of value in treating liver-localized neoplasia.

A notable advantage of encapsulating irinotecan using the methods described here is the maintenance of the drug as the active lactone form (see Table 1). Previous lipid-based techniques used to encapsulate camptothecins have exploited their ability to partition into the lipid bilayer. This has been shown to confer protection to the lactone species of irinotecan (44, 45). Therapeutically, this approach is limited by low drug loading efficiencies and the rapid exchange of membrane-localized drug from the liposomes to endogenous membranes after i.v. administration (46, 47). Tardi *et al.* (28) overcame this limitation by successfully encapsulating topotecan in the aqueous interior of liposomes using the ionophore-generated pH gradient described here. In both instances, encapsulation of topotecan and irinotecan was efficient (>90% loading). Furthermore, the internal acidic environment of the liposome limits hydrolysis of the lactone ring of camptothecins to the inactive carboxyl form. Consequently, liposomal topotecan and liposomal irinotecan are characterized by the maintenance of the lactone forms of the drugs in the circulation for extended periods after i.v. administration.

An additional facet of the liposomal irinotecan formulation described here is the steady release of drug over 24 hours. This is of particular interest in the context of improve therapeutic activity mediated by drug-carrier systems. Improvements manifested by drug carriers are often attributed to the ability of the carrier system to deliver the drug to the site of action. However, studies have demonstrated that carrier-mediated delivery of an effective agent to the tumor site is not in its self sufficient to achieve therapy. Furthermore, the concentration of drug achieved within the site of tumor growth does not necessarily predict therapeutic activity (48, 49). This apparent contradiction can be attributed to the properties of the liposomal carrier. Lipid compositions and/or encapsulation procedures selected to achieve improved drug delivery to the region of interest may hinder the release of the drug and consequently limit the amount of drug that is available to act on the target cell population.

Histologic evidence presented here (Fig. 7) indicates that this formulation accumulates in the liver, as would be expected based on experience with previous liposome preparations (50). However, 24 hours after i.v. administration, sites of liposomal localization were distinct from those of tumor growth. Moreover, the low concentrations of irinotecan associated with DSPC/Chol liposomes at this time (Table 1) suggest that the improved therapeutic activity observed does not appear to be a consequence of enhanced delivery of encapsulated irinotecan to the tumor metastases. This is supported by previous efficacy studies that demonstrated therapeutic improvements in the treatment of liver-localized disease mediated by liposomal drugs was dependent on release of the encapsulated drug from the liposomes at an appropriate rate after administration (24). It will be important to establish in future studies what release rates provide the most significant improvements in therapeutic activity.

The present study did not address how the liposome composition and irinotecan encapsulation procedure influences therapeutic activity. However, the results can be compared with other studies using comparable lipid compositions (primarily DSPC and Chol) to encapsulate doxorubicin (51), vincristine (52), mitoxantrone (53), daunorubicin (54), idarubicin (55), topotecan (28), and cisplatin (56). Such a comparison clearly demonstrates that a composition that is useful for one anticancer drug may be not be of any therapeutic value for another.

When considering the importance of drug release from liposomes in the plasma compartment or within sites of liposome accumulation, it is reasonable to suggest that the liposomal formulation used here provides sustained drug exposure over an extended time period after administration. It can be suggested, therefore, that one of the benefits of using a liposomal carrier for irinotecan is to promote prolonged tumor cell exposure to this S-phase–specific drug. This, in turn, should increase tumor growth arrest, delays in tumor progression, and increases in median survival. As discussed previously, irinotecan cytotoxicity has the additional constraint of requiring carboxylesterase-mediated conversion to the vastly more potent SN-38. These enzymes are found in high concentrations in hepatic cells, as well as in serum and other tissues, including some tumor populations (57, 58). The environment responsible for liposomal irinotecan activation and the subsequent therapeutic activity observed against colorectal metastases is currently unclear. Initial animal studies evaluated the efficacy of liposomal irinotecan in the murine LS180 and LS174T models of metastatic colorectal cancer. Although the activity of camptothecins has been evaluated, to a limited extent, in these cell lines *in vitro* (59–61), no studies have assessed irinotecan activity *in vivo* against tumors derived from these cell lines. In both the LS180 and LS174T models, liposomal irinotecan was considerably more efficacious than free drug. As shown in Fig. 5, treatment with liposomal irinotecan did not result in a decrease in tumor size, rather tumor growth was prevented for extended time periods. It can be suggested that liposomal irinotecan provides adequate control of tumor expansion but alone it is not sufficient to eradicate the disease. This is not surprising given the fact that the benefits attributable to irinotecan use have often been shown when it is used in combination with second agents such as the fluorouracils (62) and platinum-based anticancer drugs (63). In fact, irinotecan is an approved agent for treatment of colorectal cancer in combination with 5-fluoruracil plus leucovorin and is undergoing advanced testing in combination with cisplatin for treatment of lung cancer (64). We are particularly interested in developing formulations that can mediate effective delivery of irinotecan in combination with other agents selected to achieve optimal therapeutic effects as judged by assays that measure the interactions between selected drug combinations.

In conclusion, liposome encapsulation of irinotecan results in a potent drug formulation for the treatment of models of colorectal cancer as a result of increased drug longevity, protection of the active lactone species, and rapid accumulation at the site of tumor development in the liver. The use of liposomal irinotecan for the treatment of liver metastases has tremendous potential, and continuing studies focusing on optimizing the lipid formulation and understanding the role of hepatic cells in processing these carriers, as well as studies with second agents that combine well with irinotecan, could result in a chemotherapeutic strategy that will improve survival for colorectal cancer patients.

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