Development of a Highly Active Nanoliposomal Irinotecan Using a Novel Intraliposomal Stabilization Strategy

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Abstract

Liposome formulations of camptothecins have been actively pursued because of the potential for significant pharmacologic advantages from successful drug delivery of this important class of anticancer drugs. We describe nanoliposomal CPT-11, a novel nanoparticle/liposome construct containing CPT-11 (irinotecan) with unprecedented drug loading efficiency and *in vivo* drug retention. Using a modified gradient loading method featuring a sterically hindered amine with highly charged, multivalent anionic trapping agents, either polymeric (polyphosphate) or nonpolymeric (sucrose octasulfate), liposomes were capable of entrapping CPT-11 at extremely high drug-to-lipid ratios (>800 g CPT-11/mol phospholipid) and retaining encapsulated drug *in vivo* with a half-life of drug release in the circulation of 56.8 hours. CPT-11 was also protected from hydrolysis to the inactive carboxylate form and from metabolic conversion to SN-38 while circulating. The maximum tolerated dose in normal mice was determined to be 80 mg/kg for free CPT-11 and >320 mg/kg for nanoliposomal CPT-11. Nanoliposomal CPT-11 showed markedly superior efficacy when compared with free CPT-11 in human breast (BT474) and colon (HT29) cancer xenograft models. This study shows that intraliposomal stabilization of CPT-11 using a polymeric or highly charged, nonpolymeric polyanionic trapping agent results in a markedly active antitumor agent with low toxicity. (Cancer Res 2006; 66(6): 3271-7)

Introduction

Liposome-based systems have been used to enhance efficacy and/or ameliorate toxicity of certain drugs (1, 2). Thus far, the most successful approach has involved constructs engineered for long circulation times, combined with stable encapsulation of the active compound within the liposome; this allows liposomes to accumulate at sites of cancer, followed by intratumoral drug release. An example is PEGylated liposomal doxorubicin (3), which has received Food and Drug Administration approval for cancer treatment. However, the successful case of liposomal anthracyclines has not yet been matched by liposome constructs containing other anticancer drug classes, although recent progress has been made with vincristine (4–6) and certain camptothecin analogues (7–9). One of the key reasons for this has been the technical facility with which anthracyclines can be stably encapsulated in the liposome interior using remote-loading methodologies (10, 11), giving rise to stable liposome formulations that have been difficult to replicate with other classes of drugs.

CPT-11 {irinotecan; 7-ethyl-10-[4-(1-piperidino)-1-piperidino]carbonyloxycampothecin} is a water-soluble camptothecin derivative currently used in cancer chemotherapy. The pharmacology of CPT-11 is complex, with extensive metabolic conversions involved in the activation, inactivation, and elimination of the drug (12, 13). CPT-11 is a prodrug that is converted by nonspecific carboxylesterases into a 100- to 1,000-fold more active metabolite, SN-38 (14). SN-38 is cleared via glucuronidation, for which major pharmacogenetic differences have been shown (15), and biliary excretion. In addition, CPT-11 and other camptothecins exist in a pH- and serum protein-dependent equilibrium between an active lactone form of the drug (predominant under acidic conditions) and an inactive carboxylate form (predominant at neutral or basic pH; ref. 16). These drug properties contribute to the marked heterogeneities in efficacy and toxicity observed clinically with CPT-11 (12, 17). Hence, drug carrier technologies represent a rational strategy to improve the pharmacokinetics and biodistribution of CPT-11 while protecting it from premature metabolism.

In this report, we describe a novel intraliposomal drug stabilization technology for encapsulation of CPT-11 into long-circulating liposome-based nanoparticles with high drug load and high *in vivo* stability, matching or surpassing previous liposomal drugs. This was achieved using polymeric or nonpolymeric highly charged anions, polyphosphate or sucrose octasulfate, as intraliposomal trapping agents in conjunction with a high-pK_a polyalkylamine gradient. The approach also allowed for preservation of the drug in its active lactone form within the liposome interior, protecting it from hydrolysis as well as premature conversion to SN-38. Here we use the term "nanoliposomal drug" to describe a nanoparticle consisting of a lipid bilayer scaffold encapsulating a nanoscale drug complex or aggregate that facilitates *in vivo* drug retention.

Materials and Methods

Liposome Preparation and Drug Loading

Solutions of triethylammonium salts of a linear poly(phosphate) (TEA-Pn, 13-18 phosphate units; Sigma Corp., St. Louis, MO) and sucrose octasulfate (TEA_8SOS) were prepared from commercially obtained sodium salts (Toronto Research Chemicals, Inc., North York, Ontario, Canada) by ion-exchange chromatography on the Dowex 50Wx8-200 resin in the H^+ form, immediately followed by titration with neat triethylamine. Residual sodium in either solution, as determined by potentiometry using a Na^+-selective electrode, was <1% of the cation content. Phosphate content was determined by inorganic phosphate assay following acid hydrolysis and was adjusted to 0.55 mol/L for TEA-Pn (osmolality, 430-480 mmol/kg). The TEA concentration was calculated from the amount of added TEA and was

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adjusted to 0.65 mol/L for TEA_8SOS solution (osmolality, 480-530 mmol/kg). The final pH for both solutions was 5.5 to 6.0.

Distearoylphosphatidylcholine (3 mol. parts), methoxypoly(ethylene)glycol (PEG2000)-derivatized distearoylphosphatidylethanolamine (0.015 mol. parts; Avanti Polar Lipids, Alabaster, AL), and cholesterol (2 mol. parts; Calbiochem, La Jolla, CA) were combined in ~50% (w/v) ethanolic solution and mixed with 10 volumes of the solution of TEA-Pn or TEA_8SOS at 60°C to 65°C. For pharmacokinetic studies, a nonexchangeable lipid label, [^{3}H] cholesteryl hexadecyl ether (Perkin-Elmer, Boston, MA), was added to the lipids in the amount of 0.5 mCi/mmol phospholipid. The lipid suspension was extruded 15 times through two stacked polycarbonate membranes (Nucleopore, Corning-Costar, Acton, MA) with 0.08-μm pore size using argon pressure at 60°C to 65°C. The extruded liposomes were 88 to 95 nm in diameter by dynamic light scattering.

Unentrapped triethylammonium polyanions were removed by chromatography on a Sepharose CL-4B size exclusion column eluted with HEPES-buffered dextrose (5 mmol/L HEPES, 5% dextrose, pH 6.5). CPT-11·HCl (kindly provided by TTY Biopharmaceuticals, Taipei, Taiwan) was added to the liposomes at a ratio of 500 g CPT-11/mol phospholipid and the pH adjusted to 6.5. The resulting solution was heated to 60°C for 30 minutes and then quenched on ice for 15 minutes. Unencapsulated CPT-11 was subsequently removed using a Sephadex G-75 column eluted with HEPES-buffered saline (5 mmol/L HEPES, 145 mmol/L NaCl, pH 6.5). The loading efficiency was determined in all preparations by quantitating both drug and phospholipid and comparing the resulting drug/phospholipid ratio to its input value. CPT-11 was determined spectrophotometrically at 372 nm in acid/methanol (20 volume % 0.5 mol/L phosphoric acid/80 volume % methanol). Phospholipid was quantitated using a standard phosphate assay (18).

Pharmacokinetic Studies

Female Sprague-Dawley rats (190-210 g) with indwelling central venous catheters were injected with a 0.2 to 0.3 mL bolus of ^{3}H-CHE-labeled CPT-11 liposomes (10 mg/kg). Blood samples (0.2-0.3 mL) were drawn at various times postinjection using a heparin-treated syringe. The withdrawn blood volume was replaced using PBS. Blood samples were diluted with 0.3 mL of ice-cold PBS containing 0.04% EDTA, weighed, and centrifuged. Plasma was assayed for CPT-11 [by fluorometry or high-performance liquid chromatography (HPLC)] and for liposome label (scintillation radioactivity counting). The percent of drug remaining in the liposomes was calculated by dividing the drug/lipid ratio in the blood samples by that of the injected liposomes (taken as 100%). Because free CPT-11 is cleared at a much faster rate than liposomes (Fig. 3*A*), a change in the CPT-11-to-liposomal lipid ratio was indicative of drug leakage from the carrier. Noncompartmental pharmacokinetics data analysis was done using PK Solutions 2.0 software (Summit Research Services, Montrose, CO).

Drug Stability and Metabolism Studies

Liposomal and free CPT-11 were administered i.v. at a dose of 25 mg/kg in female albino rats (180-220 g) as above, and blood samples were withdrawn at intervals up to 48 hours. The blood samples were mixed with ice-cold PBS containing 0.04% EDTA and quickly centrifuged. The plasma was assayed for CPT-11, SN-38, and their carboxylate forms by HPLC using a modification of the method of Warner and Burke (19). Briefly, samples were extracted with 400 μL of ice-cold methanol by vortexing and centrifugation at 14,100 × *g* for 5 minutes. The mobile phase consisted of 3% triethylammonium acetate pH 5.5 (solution A) and acetonitrile (solution B) delivered at 1.0 mL/min in a linear gradient of 20 volume % A to 50 volume % B in 14 minutes. The eluted products were detected by fluorescence with an excitation at 375 nm and emission at 500 nm. The retention times were 5.3 minutes (CPT-11 carboxylate), 6.8 minutes (SN-38 carboxylate), 9.3 minutes (CPT-11), and 11.0 minutes (SN-38).

Conversion of CPT-11 to SN-38 was assayed in macrophages isolated from the peritoneum of female NCR *nu/nu* mice and plated at a density of 150,000 cells per well in a 12-well plate. After 24 hours, nanoliposomal CPT-11 was added to macrophages at a concentration of 10 μg CPT-11/mL and incubated for 24 hours in RPMI 1640 with 10% FCS. At indicated times, the medium was removed and the cells washed twice with Hanks buffered saline. The cells were treated with 0.2 mL of 1% Triton X-100 at room temperature for 5 minutes and solubilized in 0.8 mL of 80 volume % methanol/20 volume % 0.1 mol/L H_3PO_4 with shaking for an additional 5 minutes. The cell debris was removed by centrifugation at 13,000 rpm for 10 minutes and the supernatant was assayed by HPLC as described above.

Acute Toxicity Studies

The maximum tolerated dose following single i.v. administration was evaluated in healthy female Swiss Webster mice following a protocol adapted from the protocol communicated by the National Cancer Institute (NCI) Developmental Therapeutics Program. Briefly, in the first range-seeking step, the drug was administered via the tail vein in groups of two mice, beginning with the dose of 60 mg/kg CPT-11 and continuing with the dose escalation factor of 1.8 until acute mortality or terminal morbidity (within 1 day postinjection) was observed in any animal. The second range-seeking step was similarly done using a dose escalation factor of 1.15 and starting with the highest dose at which no mortality or terminal morbidity was observed (the highest tolerated dose) in the first step. Finally, in a validation step, a group of five mice were injected at the highest tolerated dose achieved in the second step and followed for up to 11 days for signs of general health daily and body weight twice a week. If during the observation period there was no mortality, irreversible (terminal) morbidity, or weight loss in excess of 15% of the preinjection body weight, the administered dose was considered the acute single injection maximum tolerated dose.

Antitumor Efficacy Studies

BT474 tumor model. NCR *nu/nu* athymic female mice (4-6 weeks old; Taconic Farms, Germantown, NY) were s.c. implanted at the base of tail with 60-day sustained release 0.72-mg 17β-estradiol pellets (Innovative Research of America, Inc., Sarasota, FL). Two days later, 2×10^7 BT474 human breast cancer cells were implanted s.c. in the upper back area as a 0.1-mL suspension. Tumor growth was measured by caliper along the largest (length) and smallest (width) axes twice a week. Tumor volumes were calculated using the following formula (20): tumor volume = [(length) × (width)^2] / 2. At day 13 posttumor implantation (mean tumor volume, 200 mm^3), animals were randomized to three treatment groups of 13 to 15 animals per group and treated via i.v. (tail vein) injection as described in the text. The study was continued until day 60, which also represented the duration of estrogen supplementation. Animals were weighed twice weekly. If tumors reached 20% of the mouse body weight, the animals were euthanized.

HT29 tumor model. NCR *nu/nu* athymic male mice (6-week-old, weight >16 g; Charles River, Wilmington, MA) were injected s.c. in the right flank with 0.1-mL suspensions containing 5×10^6 HT-29 human colon cancer cells. Eleven days later (mean tumor volume, 150-350 mm^3), mice were randomized to six treatment groups of 11 animals per group. Starting on day 13, the animals received four tail vein injections at intervals of 4 days of various treatments as described in the text.

Results

Preparation of nanoliposomal CPT-11. A proposed novel process using a polyalkylammonium salt of a polymeric (polyphosphate) or nonpolymeric (sucrose octasulfate) highly charged multivalent anion as intraliposomal trapping agents resulted in improvement of both the encapsulation efficiency and the *in vivo* stability of the liposome-encapsulated weakly basic, amphipathic drug CPT-11. The process may involve the formation of an intraliposomal drug-polyanion complex (Fig. 1). Sucrose octasulfate is a high-charge density molecule with one strongly acidic, negatively charged sulfate group per 1.5 carbon atoms. The triethylammonium component of the salt assists drug loading as well, ensuring the charge neutrality of the liposome interior by allowing the efflux of cations accompanying the influx of the drug and possibly by formation of a self-perpetuating pH gradient to provide a driving force for progressive drug accumulation (10).

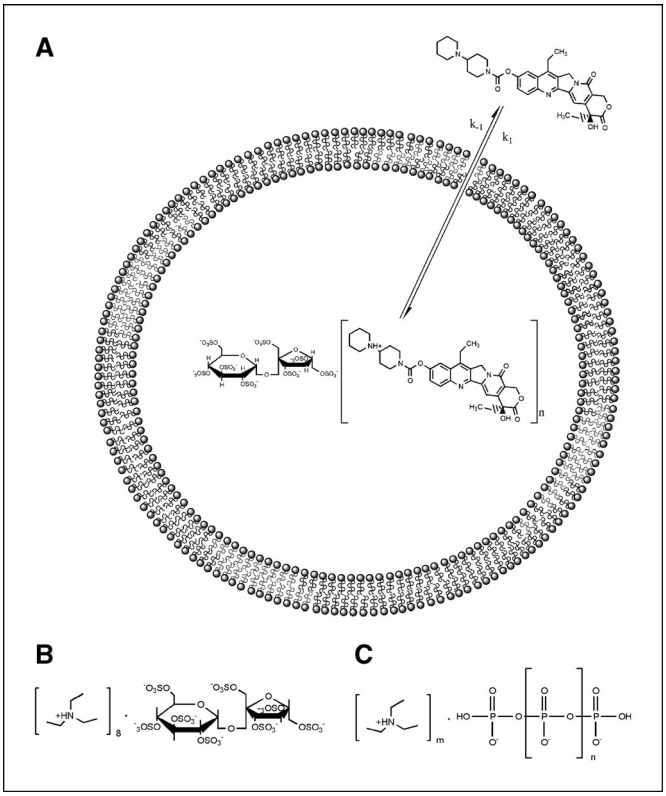

Figure 1. *A,* schematic depicting the intraliposomal stabilization strategy for CPT-11 using polyanionic trapping agents. The basic molecule CPT-11 forms a nanoscale complex with either poly(phosphate) or sucrose octasulfate in the liposome interior, thus stabilizing the liposomal formulation to increase drug retention while in circulation. Chemical structures of the triethylammonium salts of the polyanionic liposome trapping agents, poly(phosphate) (*B*) and sucrose octasulfate (*C*).

To minimize the treatment-associated lipid burden, encapsulation of CPT-11 was attempted up to drug-to-lipid ratios far exceeding the usual ratios achievable by traditional transmembrane-gradient drug loading techniques (Fig. 2). Remarkably, we found that CPT-11 encapsulation in liposomes was quantitative up to 800 g CPT-11/mol phospholipid. The final molar ratio of drug-to-phospholipid corresponds to 1.36:1 for liposomes loaded at 800 g CPT-11/mol phospholipid or 109,000 drug molecules per particle. This represents a 10- to 20-fold improvement over other liposomal formulations, including anthracyclines (3) or camptothecins lurtotecan (8) and SN-38 (21). We hypothesize that the high loading capacity of triethylammonium sucrose octasulfate liposomes is due to the formation of a stable complex between the drug and polyanion whereas the displaced triethylammonium ion dissociates and traverses the lipid bilayer as triethylamine, ensuring that the loading process continues until all added drug is encapsulated or the charge stoichiometry is achieved between the added drug and the liposomally encapsulated anion (Fig. 1).

Pharmacokinetics of nanoliposomal CPT-11. The pharmacokinetics of nanoliposomal CPT-11 formulated using either TEA-SOS or TEA-Pn were determined in normal female rats. Free CPT-11 was rapidly cleared from the circulation with $t_{1/2}$ = 0.27 hours (Fig. 3*A*). Liposome encapsulation was associated with significantly longer circulation times than free drug (Fig. 3*A* and *B*). This was especially true for liposomes loaded with TEA-SOS gradients, with blood half-lives for lipid and CPT-11 of 12.0 and 10.7 hours, respectively (Table 1).

Whereas both liposome constructs displayed long circulation for the lipid component, drug associated with TEA-SOS liposomes unexpectedly showed less rapid clearance from the blood than with TEA-Pn liposomes (Fig. 3*A* and *B*). This likely reflects that the $t_{1/2}$ of CPT-11 release from TEA-Pn liposomes was 14 hours, significantly shorter than that for TEA-SOS liposomes with a $t_{1/2}$ of CPT-11 release of 56.8 hours.

Drug stability of free and nanoliposomal CPT-11. *In vivo*, CPT-11 undergoes transformation to its more active metabolite, SN-38, and both molecules are also subject to inactivation by hydrolysis of the lactone forms to the respective carboxylate forms (Fig. 4*A* and *B*). Liposome encapsulation and delivery markedly altered these bioconversions in rats. Free CPT-11 was rapidly cleared from circulation, with only 2% of the injected dose remaining at 30 minutes and 35% of this present in the carboxylate form (Fig. 4*C*). In contrast, nanoliposomal CPT-11 showed both prolonged circulation, with 23.2% of injected dose still remaining at 24 hours, and drug protection, with no detectable conversion of CPT-11 to either SN-38 or the carboxylate form of CPT-11 (Fig. 4*D*). Thus, the high-charge density polyanionic nanoliposomal matrix provided a chaperone for the stably entrapped prodrug CPT-11, improving its pharmacokinetics and preventing its inactivation or premature conversion to the toxic metabolite SN-38.

Once deposited in tumors, liposomes are known to be taken up avidly by tumor-resident macrophages (22). To determine if macrophages could metabolically activate drug from nanoliposomal CPT-11, an *ex vivo* assay using macrophages isolated from the peritoneum of nude mice was done. Incubation of nanoliposomal CPT-11 with macrophages showed no detectable conversion to SN-38 at 24 hours but 100% conversion to SN-38 by 72 hours. This time course suggested that at least 24 hours was required for macrophage-mediated disruption of the liposome, drug release, and conversion to SN-38.

Acute toxicity of nanoliposomal CPT-11. The acute toxicity of free and nanoliposomal CPT-11 was determined in normal Swiss Webster mice using an NCI-based protocol. The maximum tolerated dose of free CPT-11 was 80 mg/kg whereas the maximum

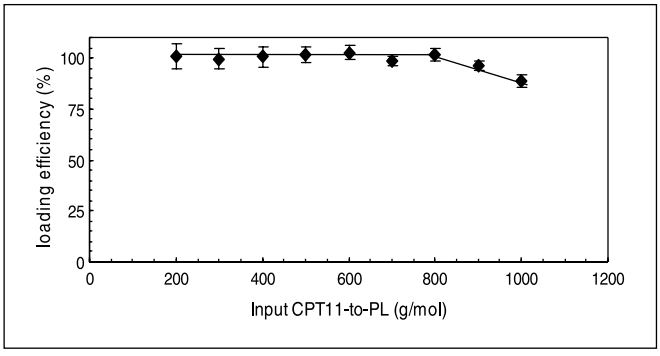

Figure 2. Liposomal loading efficiency as a function of input CPT-11-to-phospholipid (*PL*) ratio. Distearoylphosphatidylcholine/cholesterol/methoxypoly(ethylene)glycol (PEG2000)-derivatized distearoylphosphatidylethanolamine (3:2:0.015) liposomes were loaded with CPT-11 as described in Materials and Methods. The resulting CPT-11-to-phospholipid ratio following loading was determined by quantitating both CPT-11 and phospholipid in the resulting purified liposomal CPT-11 formulation, and the loading efficiency by comparing this ratio to the input ratio.

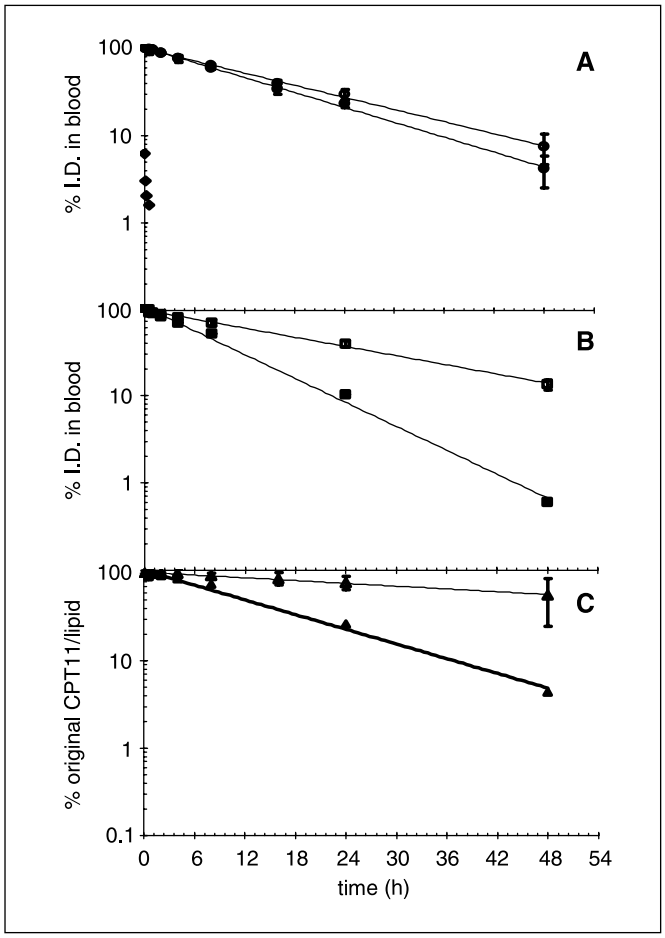

Figure 3. Pharmacokinetics of nanoliposomal CPT-11 in rats. Nanoliposomal CPT-11 prepared using either triethylammonium sucrose octasulfate (*A*) or poly(phosphate) (*B*) was administered i.v. in 9-week-old female Sprague-Dawley rats (body weight ~200 g) with indwelling central venous catheters at a dose of 10 mg CPT-11/kg (17.6 μmol phospholipid/kg). Free CPT-11 was administered i.v. as a bolus injection at 25 mg/kg (*A*, ♦). Plasma was sampled at the indicated times and analyzed for drug and liposomal lipid content. *Points,* % of injected dose (*% I.D.*) of lipid (○, □) or drug (●, ■, ♦). *C,* drug retention was calculated as percent of original drug associated with liposomal lipid at each time point for the poly(phosphate) (▲) and sucrose octasulfate (△) formulations.

tolerated dose of nanoliposomal CPT-11 formulated using a TEA-SOS gradient was not achieved even at the highest administered dose of 324 mg CPT-11/kg. A dose of >324 mg CPT-11/kg was impossible to administer because of concentration and injection volume limitations. Therefore, nanoliposomal CPT-11 delivery reduced drug toxicity in the mouse by at least 4-fold.

Efficacy of nanoliposomal CPT-11 in the BT474 breast cancer model. Treatment using nanoliposomal CPT-11, formulated using the TEA-Pn loading strategy, was evaluated in the BT474 breast tumor xenograft model (Fig. 5*A*). Free CPT-11 was clearly efficacious in this model with noticeable inhibition of tumor growth. However, treatment with nanoliposomal CPT-11 provided further advantage with dramatic regressions in tumor volumes and 100% cures of mice (defined as no residual tumor at study end).

Treatment-related toxicities were not observed. There was a slight decrease in mean body weight by 3.3% on the final treatment day in the animals receiving liposomal CPT-11; this decrease was not statistically significant compared with pretreatment weight ($P = 0.274$, Student's t test). All other weight measurements were within the expected range.

Efficacy of liposomal CPT-11 in the HT29 colon cancer model. In the HT29 colon tumor xenograft model, free CPT-11 again showed efficacy, albeit modest (Fig. 5*B*). However, both nanoliposomal CPT-11 formulations showed pronounced antitumor effects, including tumor regression during treatment followed by prolonged absence of tumor regrowth. Indeed, at 42 days postimplantation, all nanoliposomal CPT-11 treatments seemed to be equivalent and maximally efficacious.

With continued observation, tumor regrowth was observed beginning on day 47 postimplantation. At this point, all control and free CPT-11-treated mice had been sacrificed due to excessive tumor growth. Based on regrowth rates, treatment with TEA-SOS liposomes was more efficacious than TEA-Pn liposomes administered at the same CPT-11 dose. Furthermore, treatment with either liposome type at 50 mg/kg dose was more efficacious than at 25 mg/kg. In an analysis of cure rates, no mice receiving control or free CPT-11 were cured. Mice receiving TEA-Pn liposomal drug at 50 mg/kg per injection, despite initial tumor regressions, showed eventual regrowth. In the two groups receiving 25 mg/kg of either liposome formulation, one animal (9.1%) from each group was tumor-free at study end. In the group receiving 50 mg/kg of the TEA-SOS liposome formulation, 4 animals (36.4%) showed no regrowth and remained tumor-free.

Animals receiving free CPT-11, but not any of the nanoliposomal CPT-11 preparations, showed morbidity (loss of alertness, humped posture, ruffled fur, decreased mobility) for 1 hour after drug injection. Animals receiving free CPT-11 also lost 6% of weight

Table 1. Pharmacokinetic variables for free and nanoliposomal CPT-11 in rats

Formulation	$t_{1/2}$ (h)	AUC_∞ (μg h/mL)	CL (mL/h)	V_d (mL)	MRT (h)	$t_{1/2}$ CPT-11 release (h)
Free CPT-11	0.27	6.2	1,609	616.4	0.4	—
Ls-CPT-11 [TEA-Pn]	6.80	1,407.8	7.10	69.7	9.8	14.0
Ls-CPT-11 [TEA-SOS]	10.7	2,134.4	4.69	72.3	15.4	56.8

NOTE: The data used to calculate the pharmacokinetic variables for CPT-11 when formulated either in the free form or liposomal form refer to the actual drug concentrations measured in the blood that were then used to calculate the %ID values found in the corresponding curves for Fig. 3*B*.
Abbreviations: AUC_∞, area under the concentration versus time curve in plasma based on the sum of exponential terms; MRT, mean residence time calculated from exponential terms; CL, clearance calculated from exponential terms; V_d, volume of distribution.

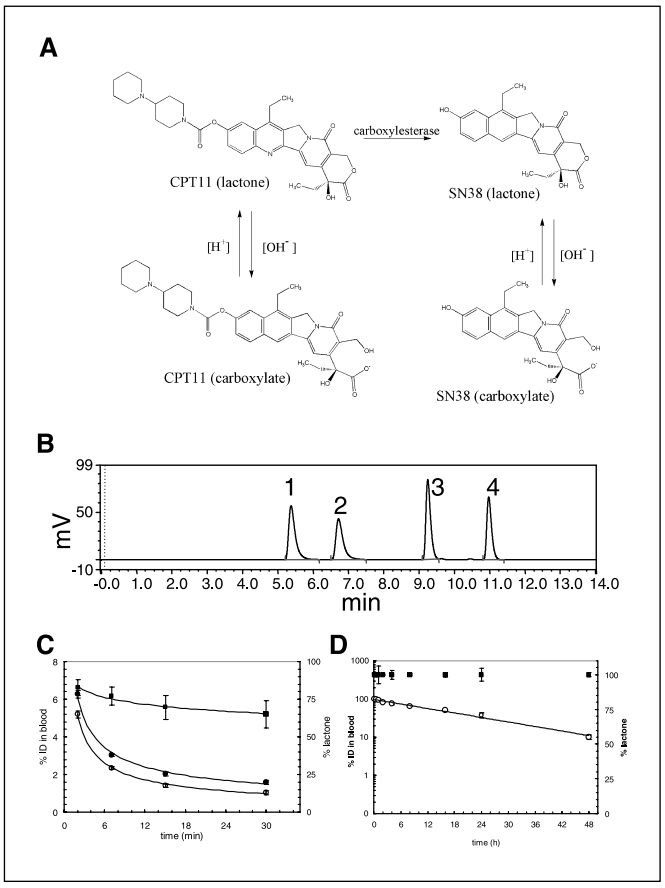

Figure 4. Drug stability of free and nanoliposomal CPT-11. *A,* CPT-11 and SN-38 exist in a pH-dependent equilibrium between closed lactone and open carboxylate configurations. CPT-11 is converted to its more active metabolite, SN-38, by carboxylesterases. *B,* HPLC chromatogram showing the separation of these species: CPT-11 carboxylate (*peak 1*), SN-38 carboxylate (*peak 2*), CPT-11 lactone (*peak 3*), and SN-38 lactone (*peak 4*). The *in vivo* drug stability of free (*C*) and nanoliposomal (*D*) CPT-11 was evaluated following single i.v. bolus administration at a dose of 25 mg CPT-11/kg in 9-week-old female Sprague-Dawley rats (body weight ~200 g). Levels of total CPT-11 (●) and CPT-11 lactone (○) in the blood were determined by HPLC analysis and expressed as percent of initial CPT-11 dose. *Right y axis,* percentage of CPT-11 in the lactone form is plotted as a function of time (■).

during treatment and did not recover, probably because of the effects of the growing tumor. Animals receiving nanoliposomal CPT-11 formulations experienced transient weight loss of 5% (at 25 mg/kg) or 9% (at 50 mg/kg) between the second and third injections as compared with pretreatment values; however, weights recovered following completion of treatment.

Discussion

Liposome delivery has been shown to improve the pharmacokinetic profile and widen the therapeutic index of certain anticancer drugs, especially the anthracycline class (1, 2). Improved efficacy is in part a result of passive targeting to tumor sites based on the enhanced permeability and retention (EPR) effect (23). To fully exploit this process, drug carriers must be engineered to retain drug while circulating, thereby preventing premature drug release before accumulating in the tumor but still allowing for release of drug once in the vicinity of the tumor. Antibody-targeted nanoparticles, such as immunoliposomes against HER2 (24) or epidermal growth factor receptor (25), represent another strategy for more efficient drug delivery to tumor cells.

Gradient-based drug loading technologies, in which electrochemical gradients drive the accumulation of drugs in the liposome interior, represent a key advance in liposome research (11, 26). This approach was further refined when transmembrane gradients of ammonium ion were proposed to form a self-sustaining pH-gradient that can load drugs inside liposomes (10). However, weakly basic anthracyclines represented the only drug class that afforded slow *in vivo* release rates when loaded using gradients involving common anionic counterions, such as sulfate or citrate. With other drug classes, gradient-based loading has been achieved with variable efficiency. To stabilize other cationic drugs against premature escape from liposomes, the use of pre-entrapped polyanionic polymers was proposed (9, 27).

In the present study, we used a drug loading transmembrane gradient system with two components, a substituted ammonium and a poly(anionic) trapping agent of either polymeric (polyphosphate) or nonpolymeric (sucrose octasulfate) nature. The use of polymeric polyanions such as heparin or dextran sulfate to improve liposomal drug retention has been reported (9, 27). Polyphosphate was effective in stabilizing intraliposomal CPT-11

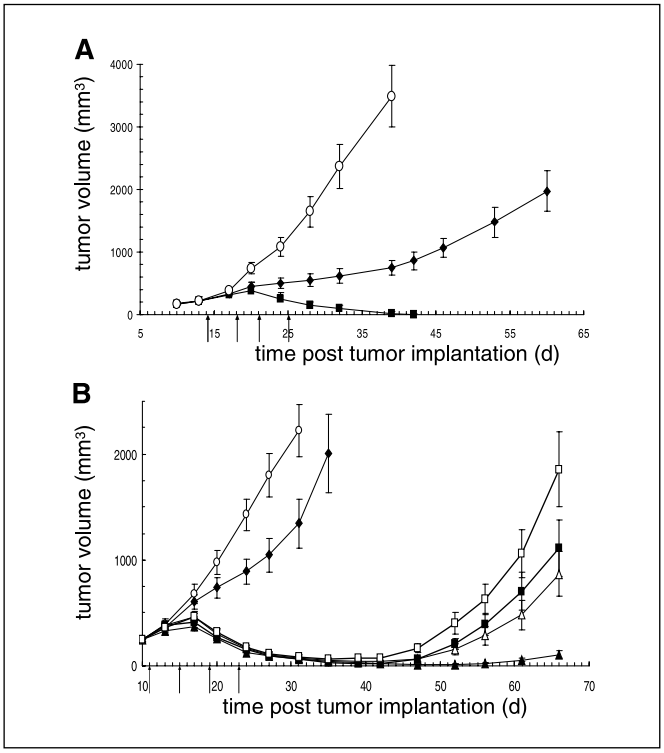

Figure 5. Antitumor efficacy of nanoliposomal CPT-11 in tumor xenograft models. *A,* BT474 breast cancer cells were implanted s.c. in nude mice along with estrogen pellets. When tumors were well established and had reached mean volumes of 200 mm^3, the following treatments were initiated: control (○), drug- and liposome-free vehicle only; free CPT-11 (♦); or nanoliposomal CPT-11 stabilized with TEA-Pn (■). Free and nanoliposomal CPT-11 were injected at 50 mg CPT-11/kg/dose i.v. twice per week for four doses (*arrows*). *B,* HT-29 colon cancer cells were implanted s.c. in nude mice. When tumors were well established and had reached mean volumes of 150 to 350 mm^3, the following treatments were administered: control (saline; ○); free CPT-11, 50 mg/kg/dose (♦); nanoliposomal CPT-11 using TEA-Pn, 25 mg/kg/dose (□); nanoliposomal CPT-11 using TEA-Pn, 50 mg/kg/dose (■); nanoliposomal CPT-11 using TEA-SOS, 25 mg/kg/dose (△); and nanoliposomal CPT-11 using TEA-SOS, 50 mg/kg/dose (▲).

against *in vivo* release, having the added advantage of being more readily biodegradable than dextran sulfate. However, polyanionic polymers such as heparin and dextran sulfate have notable anticoagulant activity and, in the case of dextran sulfate, toxic to Kupffer cells (28). The undefined chemical nature of many functionalized polymers may also contribute to variability in *in vivo* properties. Unexpectedly, we observed that a highly charged, nonpolymeric anion, such as sucrose octasulfate, provided even better drug retention than a polyanionic polymer, resulting in outstanding *in vivo* drug encapsulation stability. Sucrose octasulfate is a product of exhaustive esterification of sucrose, using chlorosulfonic acid or sulfur trioxide in pyridine or methylpyridine, and is a known pharmaceutical ingredient, the basic aluminum salt (Sucralfate) of which is widely used to treat gastric hyperacidity (29). Compared with dextran sulfate, sucrose octasulfate is chemically well defined; it does not have known anticoagulant or antimacrophage activity (29) and its salts can be produced in pure crystalline form ensuring less interlot variability.

The concept of nanoparticle delivery of camptothecins is very attractive based on potential advantages, including overcoming the solubility limitations of this class, protecting drug in the active lactone configuration, rerouting of drug from sites of toxicity such as the gastrointestinal tract, prolonging circulation time, increasing tumor accumulation via the EPR effect, and providing sustained release for a so-called metronomic effect. Using a novel intraliposomal stabilization technology, we have developed a nanoliposomal CPT-11 featuring drug loading efficiency and drug payload ($>10^5$ per particle) in far excess of that previously reported for this type of encapsulation; this agent showed marked *in vivo* retention of CPT-11 during long circulation times while simultaneously protecting the drug from lactone hydrolysis or premature activation. Compared with free CPT-11, this liposome-based nanoparticle reduced host toxicity in rats by >4-fold and greatly increased antitumor efficacy in animal models. In a separate study, we showed similar improvements in efficacy and host toxicity when nanoliposomal CPT-11 was administered locally to brain tumors using convection-enhanced delivery (30).

Previously reported liposomal camptothecin preparations have shown increased efficacy but not necessarily improved toxicity when compared with free drug (8, 9, 31). Other examples have shown prolonged circulation (32, 33), but not to the extent observed for the TEA-SOS-stabilized liposomes described here. In addition, a liposomal version of SN-38 is cleared even more rapidly with an AUC_∞ that seems to be at least 2 orders of magnitude less than that observed for nanoliposomal CPT-11 (34).

Another aspect of nanoliposomal CPT-11 is that it delivers a prodrug. Cytotoxic drugs encapsulated in liposomes are normally unable to act on their therapeutic targets or cause toxicity until they can be released from the confines of the carrier, and thus liposomal drug delivery can itself be regarded as a prodrug strategy. Hence, in this dual prodrug strategy, liposome delivery of CPT-11 chaperones the camptothecin until it reaches tumor sites where the prodrug can then be activated locally. Although local activation of CPT-11 to SN-38 has yet to be shown, carboxylesterases have a widespread distribution in different tumor types (35–37) and are active in macrophages, the principal scavenger of liposomes. Indeed, we observed that nanoliposomal CPT-11 was completely converted to SN-38 by macrophages after 72-hour incubation. We hypothesize that nanoliposomal CPT-11 may be acted on by tumor-resident macrophages, which convert drug to SN-38 with subsequent diffusion to nearby tumor cells. Alternatively, CPT-11 may be activated directly by tumor cells following release from its liposome carrier.

We conclude that nanoliposomal CPT-11 generated by novel intraliposomal drug stabilization resulted in advantageous pharmacologic properties with increased efficacy and reduced host toxicity *in vivo*. The drug-loading and stabilization technologies used for CPT-11 may also be broadly applicable to other weakly basic anticancer drugs as we have recently shown using a novel histone deacetylase inhibitor, LAQ824 (38). Nanoliposomal CPT-11 may provide a robust and useful nanoparticle-based treatment for cancer.

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