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Development of a weak-base docetaxel derivative that can be loaded into lipid nanoparticles

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ABSTRACT

Hydrophobic uncharged drugs such as docetaxel are difficult to encapsulate and retain in liposomal nanoparticles (LNP). In this work we show that a weak base derivative of docetaxel can be actively loaded into LNP using pH gradient loading techniques to achieve stable drug encapsulation and controlled release properties. Docetaxel was derivatized at the hydroxyl group in the C-2′ position to form an N-methyl-piperazinyl butanoic acid ester. The free hydroxyl group in this position is essential for anticancer activity and the prodrug has, therefore, to be converted into the parent drug (docetaxel) to restore activity. Cytotoxicity testing against a panel of cancer cell lines (breast, prostate and ovarian cancer) demonstrated that the prodrug is readily converted into active drug; the derivative was found to be as active as the parent drug in vitro. The docetaxel derivative can be efficiently loaded at high drug-to-lipid ratios (up to 0.4 mg/mg) into LNP using pH loading techniques. Pharmacokinetic, tolerability and efficacy studies in mice demonstrate that the LNP-encapsulated prodrug has the long drug circulation half-life required for efficient tumor accumulation (50–100 times higher drug plasma levels compared with free derivative and Taxotere™, the commercial docetaxel formulation), is active in a xenograft model of breast cancer (MDA-MB-435/LCC6), and is well tolerated at i.v. doses of 3 times higher than the maximum tolerated dose (MTD) of the parent drug. This is the first demonstration that a therapeutically active, remote-loaded, controlled-release LNP formulation of a taxane can be achieved. The approach reported here has broad applicability to other approved drugs as well as new chemical entities.

1. Introduction

Liposomal nanoparticles (LNP) are the leading drug delivery systems for the systemic (intravenous) administration of anticancer, antimicrobial and anti-inflammatory drugs. Seven liposomal drugs have received regulatory approval and many more are in clinical trials [1–3]. This success is largely due to the ability of long-circulating LNP to increase the effectiveness of chemotherapeutic agents by delivering high concentrations of the drug to disease sites, while reducing the toxicity of the drugs by reducing their accumulation in normal tissues [1–4]. The increased delivery to disease sites is due to the enhanced extravasation of nanoparticles through the "leaky" vasculature in such regions. In the case of solid tumors, this can result in 100-fold or more improvements in the amount of drug delivered to the tumor. This has resulted in significant improvements in the drug's therapeutic index that extended from animal studies through to the clinic [1,3,4].

The therapeutic benefits of the LNP technology rely on the ability to efficiently load the drug into the liposomal carrier and to retain it in the carrier so that it is delivered to the disease site, and once there released at an appropriate rate. This is readily achieved for drugs that are weak bases (which include many anticancer drugs), which can be loaded into LNP at high concentrations in response to a transmembrane pH gradient [2,5,6]. However, there are important anticancer drugs that do not have the weakly basic amino groups that are required for drug loading and they cannot, therefore, be stably encapsulated and retained in LNP. This is exemplified by the taxanes.

The taxanes, including paclitaxel and docetaxel, are one of the most important families of anti-cancer drugs. They have broad activity and are extensively used in clinical oncology [7,8]. The compounds exhibit poor water-solubility, which has to be overcome for the development of clinically viable formulations. This is difficult to achieve. Initial formulation approaches were focused on the use of

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surfactants such as Cremophor and polysorbate 80, which led to the development of Taxol™ (paclitaxel) and Taxotere™ (docetaxel), respectively, which are currently the most widely prescribed anticancer drugs on the market. These commercial formulations are associated with a number of serious pharmacological and toxicological concerns, including dose-limiting myelosuppression (neutropenia), neurotoxicity, large volumes of distribution, highly variable (docetaxel) and non-linear (paclitaxel) pharmacokinetics (PK) as well as toxicity (hypersensitivity reactions) associated with the formulation vehicle (Cremophor EL, polysorbate 80) [8–13]. In the case of Taxotere™, the large variability in PK causes significant variability in toxicity and efficacy and the hematological toxicities are correlated with systemic exposure to free (unbound) drug [10]. These problems can potentially be overcome through use of appropriately designed delivery systems such as lipid-based nanoparticles (LNP). Current strategies to develop LNP-based formulations of paclitaxel and docetaxel have focused on incorporation of these drugs in the lipid bilayer of LNP [14–18]. However, these formulations are not stable in the blood circulation; drug rapidly exchanges from the LNP bilayer and no clinical benefit of such LNP formulations over the established taxane formulations has been observed [17,18]. The LNP solely functions as a solubilizing agent similar to the surfactants used in the conventional formulations; however, the lipids are less toxic.

In this publication a new approach is described that extends the benefits of LNP technology to drugs such as docetaxel and paclitaxel for which stable LNP (and other) formulations can normally not be achieved. A protonable weak base docetaxel derivative that can be efficiently loaded and retained in LNP has been synthesized. LNP encapsulation dramatically extended drug circulation half-life from minutes to hours compared to docetaxel/Taxotere™ and increased the maximum tolerated dose by more than 3-fold. This results in significantly better anticancer activity *in vivo* compared to Taxotere™, the commercial docetaxel formulation. The results of this work demonstrate the pharmaceutical potential of the novel LNP-docetaxel formulation and validate the new approach.

2. Materials and methods

2.1. Lipids and chemicals

Docetaxel was purchased from BioxelPharma (Québec, Canada). Cholesterol (Chol), distearoylphosphatidylcholine (DSPC), dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylcholine (DMPC)) were from Avanti Polar Lipids (Alabaster, AL). Tritiated [^{3}H] cholesteryl hexadecyl ether ([^{3}H]CHE) was purchased from Perkin-Elmer (Boston, MA). Taxotere™ for *in vivo* studies (PK, tolerability and efficacy) was purchased from the BC Cancer Agency Pharmacy. All other chemicals were obtained from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). All reagents were analytical or HPLC grade and were used without further purification. Water was prepared by a reverse osmosis system (MilliQ) and filtered through a 0.2 μm filter prior to use.

2.2. Mice

Female Swiss Webster (Simonsen Laboratories Inc, Gilroy, CA) were used for the pharmacokinetic/*in vivo* drug release studies and Rag2-M (BC Cancer Research Centre, Vancouver, BC) mice for the tolerability/dose range finding study and the anticancer efficacy studies. All mice (6–8 weeks old) were quarantined for at least 2 weeks prior to use. Animals were maintained in a controlled temperature and humidity environment. Lighting was maintained on automatic 12 h light/dark cycles. All animal studies were conducted in compliance with the guidelines established by the Canadian Council on Animal Care (CCAC).

2.3. Synthesis

Docetaxel was esterified at the 2′-hydroxyl group with 4-(4-methylpiperazin-1-yl)butanoic acid to form a prodrug as described in detail in the Supplementary Data. The syntheses of both the requisite amino acid and esterified prodrug are straightforward and have been carried out on multigram scale. The prodrug was isolated as the hydrochloride salt with purities (by UHPLC-UV) of >96%. Identity, purity and composition of the compound were confirmed by 1D and 2D ^{1}H- and ^{13}C-NMR, UHPLC-MS and elemental analysis (see Supplementary Data).

2.4. Preparation of LNP

LNP were prepared based on the ethanol procedure described by Boman et al. [19]. Briefly, lipids (phospholipid/Chol, 55/45 molar ratio) were dissolved in ethanol and added slowly into an aqueous solution containing 350 mM ammonium sulfate at 60 °C; trace amounts of the lipid marker [^{3}H]CHE (0.15 μCi/mg total lipid) were co-dissolved with the other lipids in ethanol to prepare LNP for release studies. The final ethanol concentration was 15% (v/v). The resulting LNP dispersions were extruded at 60 °C through two stacked 100 nm polycarbonate filters (Nuclepore, Pleasanton, CA) using a heated thermobarrel extruder (Northern Lipids, Vancouver, Canada), as described by Hope et al. [20]. Residual ethanol and external ammonium sulfate were removed by tangential flow diafiltration at room temperature, and replaced with a 300 mM sucrose solution using a Midgee™ HOOP™ ultrafiltration cartridge (MW cut-off 100000; Amersham Biosciences). LNP were concentrated by diafiltration to the desired concentrations. Quasi-elastic light scattering (QELS) was used to assess the size distribution of the extruded LNP (target size 100 ± 20 nm), using a NICOMP model 370 submicron particle sizer (Particle Sizing Systems, Santa Barbara, CA).

2.5. Drug loading

The docetaxel derivative was loaded into LNP using the ammonium sulfate-based loading method described by Haran et al. [21]. Briefly, the docetaxel derivative was dissolved at 2 mg/mL in 300 mM sucrose and added to pre-heated (60 °C) LNP suspensions followed by incubation under stirring at 60 °C for the indicated times (typically 30 min). The formulations would typically be prepared at lipid concentrations between 5 and 10 mg/ml and drug-to-lipid weight ratios of 0.1–0.4 mg/mg. The unencapsulated docetaxel derivative was removed by tangential flow diafiltration using a Midgee™ HOOP™ ultrafiltration cartridge (MW cut-off 100000; Amersham Biosciences), and the external solution replaced with non-buffered physiological saline solution and the sample concentrated by diafiltration as needed. Loading efficiencies were determined by quantitating both prodrug and lipid levels before and after separation of external prodrug from LNP encapsulated prodrug by size exclusion chromatography using Sephadex G-50 spin columns and comparing the respective prodrug/lipid ratios. Phospholipid concentrations were determined by the phosphorus assay of Fiske and Subbarow [22], cholesterol was quantitated by an enzymatic colorimetric method employing a standard assay kit (Wako Chemicals, Richmond, VA). Docetaxel derivative was determined by ultra high pressure liquid chromatography (UHPLC) as described below. The drug-loaded LNP formulations for *in vivo* studies were sterilized by filtration through 0.2 μm filters (Nalgene) and subsequently stored at 4 °C.

2.6. Cryo-transmission electron microscopy (cryo-TEM)

The Cryo-TEM studies were performed with a Tecnai G2 20 TWIN Mk. 2 Transmission Electron Microscope (UBC BioImaging Facility, Vancouver, Canada). The instrument was operating at 200 kV in

bright-field mode. Digital images were recorded under low dose conditions with an FEI Eagle 4 k HR CCD camera and analysis software FEI TIA. An underfocus of 1–3 μm was used to enhance image contrast. Sample preparation was done with a Vitrobot Mark IV vitrification robot on Lacey Formvar 300 grids #01890.

2.7. Analytical and bioanalytical assays for docetaxel and docetaxel derivative

Docetaxel and docetaxel derivatives were quantitated by ultra high pressure liquid chromatography (UHPLC). The instrument consisted of a Waters® Acquity™ UPLC system equipped with a photodiode array detector (PDA) and a triple-quad (TQ) MS detector; Empower™ data acquisition software version 2.0 was used (Waters, USA). Separations were performed using a Waters® Acquity™ BEH C18 column (1.7 μm, 2.1×100 mm) at a flow rate of 0.25 mL/min, with mobile phases A and B consisting of water with 0.1% trifluoroacetic acid (TFA) and acetonitrile with 0.1% TFA, respectively. The mobile phases were delivered at a programmed linear gradient at a column temperature of 23 °C. Separation was initiated with a mobile phase ratio of 50:50 (A:B). The ratio was changed to 10:90 (A:B) over a period of 2 min using a linear curve and then maintained at 10:90 (A:B) over a period of 0.5 min. The mobile phase was subsequently changed back to 50:50 (A:B) over a period of 0.1 min and this ratio was maintained for 0.4 min before the next sample was injected. The analyte was detected by a PDA and TQ-MS detector at a wavelength of 230 nm and ES^+ ion mode with a cone voltage of 30 V, respectively. The limit of MS quantitation (LOQ) was 0.05 μg/mL for the derivative and 0.5 μg/mL for docetaxel. For assaying the docetaxel and docetaxel derivative in LNP formulations, aliquots of the latter were solubilized in TFA-acidified ethanol (0.1% vol). For docetaxel and docetaxel derivative detection in plasma samples, 50 μL plasma was added to 150 μL ice-cold methanol acidified with TFA (0.1% v/v) and the mixture was centrifuged at 4 °C for 30 min at 10,000 g to pellet the precipitated proteins. Acidification of methanol was found necessary to stabilize the prodrug. The supernatant was removed and aliquots were analyzed as described above using MS detection. The recovery of the prodrug and docetaxel from plasma was >92%.

2.8. Chemical stability in aqueous solutions and plasma

The chemical stability of the prodrug was determined in aqueous solutions at different pH values and temperatures as well as in biological media such as mouse plasma. Aliquots of docetaxel derivative in water were mixed with buffer (citrate/HEPES, 10 mM/10 mM, pH 4.0, 7.5 or 8.0) or mouse plasma in 1 mL glass HPLC sample vials sealed with Teflon-lined caps (final volume 0.25 ml, final docetaxel derivative concentration 50 μg/ml) and incubated for 24 h. Buffer stability was determined at three different temperatures (4 °C, RT and 37 °C); plasma stability was determined at 37 °C. The mouse plasma was buffered with 50 mM phosphate buffer (50:50 v/v) to keep the pH constant at 7.4 during the 24 h duration of the experiment. At desired time points, the content of the vials was mixed with 0.75 ml of methanol acidified with 0.1% TFA and analyzed by UHPLC as described above.

2.9. Solubility

Ten milligrams of derivative was weight into a glass vial and then 2 mL of 5 mM citrate buffer (pH 4) or 10 mM acetate buffer (pH 5) was added followed by sonication of the suspension for 10 min and 20 min incubation at RT. The precipitate was then removed using Microcon MY-100 centrifugal filters (MW cut-off 100,000 Da) and the filtrate analyzed by UHPLC-UV for drug content.

2.10. In vitro drug release

In vitro release of the docetaxel derivative from LNP was determined in mouse plasma by monitoring changes in the prodrug/lipid ratio as a function of time. LNP were prepared containing trace amounts of the radiolabeled lipid marker ^{3}H-cholesterylhexadecylether ([^{3}H]-CHE). LNP dispersions were loaded with the prodrug and then mixed with mouse plasma at a final lipid concentration of 0.75 mg/ml, followed by incubation at 37 °C. At various time points, aliquots were taken and run over Sephadex G-50 spin columns to remove the unentrapped prodrug [23]. The prodrug and lipid concentrations in the eluates were determined by UHPLC and liquid scintillation counting, respectively. The percentage of retention was defined as the prodrug/lipid ratio found in the sample taken at a specified time point divided by the initial drug-to-lipid ratio.

2.11. Formulation stability

LNP-prodrug formulations with different lipid compositions (DSPC/Chol, DPPC/Chol and DMPC/Chol all at 55/45 mol% and a prodrug/lipid ratio of 0.2 wt/wt) were prepared at a prodrug concentration of 3 mg/ml in 0.9% physiological saline, sterile filtered and sterile-filled into 5 mL glass vials; the vials were stoppered and capped and then stored in the fridge at 7 °C. At various time points, formulations were analyzed for size (QELS), prodrug retention (Sephadex G-50 spin column method) and prodrug integrity as described above.

2.12. In vitro anticancer activity

The Alamar Blue assay [24,25] was used to compare the cytotoxic activity of derivatized docetaxel to that of docetaxel in a panel of human cancer cell lines including the clear cell carcinoma cell line ES-2, the castration insensitive prostate cancer cell line PC3 and the breast cancer cell line MDA435/LCC6 (BC Cancer Agency, Vancouver, BC). Cells were incubated in 96 well plates at 37 °C for 72 h in the presence of varying amounts of the derivative or the parent drug (dissolved in DMSO); at the end of the incubation period, Alamar Blue solution was added to all of the wells (20 μl/well, 10% of culture volume). The plates were returned to the incubator for 4 h; sample fluorescence was determined at $\lambda_{ex}=530$ nm and $\lambda_{em}=590$ nm. Viability was calculated according to: Cell viability (%) $=(F_{\text{plus drug}}-F_{\text{background}})/(F_{\text{minus drug}}-F_{\text{background}})*100$, where $F_{\text{plus drug}}$ is the fluorescence reading in the presence of drug, $F_{\text{minus drug}}$ the cell control in the absence of drug and $F_{\text{background}}$ the background fluorescence (media alone). IC_{50} values (nM) were calculated by fitting a sigmoidal curve to the concentration-viability plot.

2.13. Pharmacokinetics and in vivo drug release

The PK of LNP-encapsulated derivative was compared to that of Taxotere™, the commercial docetaxel formulation and the derivative formulated in the same manner as Taxotere™. Taxotere™ was reconstituted as described in the prescribing information [8]. The docetaxel derivative was formulated in the same way as Taxotere using ethanol/polysorbate 80/physiological saline solution to dissolve the drug. The docetaxel derivative was encapsulated in DSPC/chol, DPPC/Chol and DMPC/Chol LNP (55:45 mol%) containing trace amounts (0.15 μCi/mg lipid) of the lipid marker [^{3}H]CHE at a prodrug-to-lipid ratio of 0.2 wt/wt using the ammonium sulfate loading technique. This allows to follow both the elimination of the prodrug as well as that of the LNP carrier from circulation. The PK and *in vivo* release studies were based on 4 time points (1, 4, 8 and 16 h) and 4 mice per time point. All formulations were administered i.v. via the lateral tail vein at docetaxel (or equivalent docetaxel) doses of 20 mg/kg and a volume based on the individual mouse's weight

(10 mL/kg). At various times, mice were anesthetized with ketamine/xylazine, blood collected by cardiac puncture and placed into EDTA microtainer tubes. Animals were terminated immediately after blood collection. Plasma was separated from whole blood by centrifugation at 1000 *g* for 10 min. Plasma proteins were precipitated as described above in bioanalytical assays and the supernatant analyzed for docetaxel and derivative by UHPLC. In the case of the LNP formulations, 25–50 µl of plasma was added to scintillation fluid (PicoFluor 40, Perkin Elmer) and analyzed for lipid levels ([^{3}H]-CHE radioactivity) by scintillation counting. The percentage of prodrug remaining in LNP (drug retention) was calculated by dividing the prodrug/lipid ratios found in plasma samples by those of the injected LNP formulations, taken as 100%. As free docetaxel and docetaxel derivative were cleared at much faster rate than in LNP-encapsulated form, the prodrug/lipid ratios recovered from the plasma samples can be regarded as a direct indication of the amount of prodrug remaining encapsulated in LNP.

2.14. Tolerability in mice/dose range finding study

Tolerability studies were performed in female Rag2-M mice with LNP-encapsulated docetaxel derivative, docetaxel derivative (formulated in the same manner as docetaxel in Taxotere™) and Taxotere™. The studies were based on administration of a single dose and relied on 3 mice/group and a dose escalation strategy based on three dose levels for the derivative and Taxotere and five dose levels for the LNP formulations (DSPC/Chol 55:45 mol% at a prodrug/lipid weight ratio of 0.2 mg/mg). All formulations were injected i.v. via the lateral tail vein in a volume of 200 µl/20 g mouse. Mice were monitored daily for signs of toxicity over a period of 14 days following drug administration. Body weights of individual mice were measured every second day over the course of the study. The MTD of the different formulations was estimated as the dose that results in ~15% loss in body weight and does not cause lethality. If weight loss was not a good predictor of tolerability, the dose where no animals needed to be terminated due to toxicity was used.

2.15. Efficacy studies in MDA-MB-435/LCC6 (breast cancer) xenograft model

MDA-MB-435/LCC6 cells were provided by Dr. Robert Clarke (Georgetown University) and cultured in DMEM with 2 mM L-glutamine and 10% FBS at 37 °C in 5% CO_2 environment. Female RAG2-M mice were inoculated with 5×10^6 (50 µL) cells subcutaneously on the right hind flank. Once tumors reached a size of 100–150 mm^3, animals were randomized into groups (6 animals per group) and injected with a single i.v. bolus injection of Taxotere™ at a dose of 25 mg/kg or LNP formulations of docetaxel derivative (DSPC/Chol, DPPC/Chol and DMPC/Chol at 55:45 mol% and a prodrug/lipid weight ratio 0.2 wt/wt) at three different doses (31.25 mg/kg, 50 mg/kg and 110 mg/kg, which is corresponds to 25, 40 and 88 mg/kg docetaxel). Tumor growth and animal weights were measured every third day. Tumor growth was monitored by measuring tumor dimensions with digital calipers and tumor volumes were calculated according to the equation length$\times$(width^2)$\div$2 with the length (mm) being the longer axis of the tumor. Tumors were allowed to grow to a maximum of 700 mm^3 before termination; animals with ulcerated tumors were terminated. The effectiveness of the treatment was assessed through comparison of established parameters of anticancer activity including: tumor growth inhibition (optimal %T/C); tumor growth delay (T-C), difference in time for treated and control tumors to double in size; and NCI score [26]. Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA). A one-way ANOVA (Analysis of Variance) with Tukey's multiple comparison test was used to compare tumor growth data. The threshold significance level was set at 0.05 ($p<0.05$ was considered statistically significant).

3. Results

3.1. The docetaxel prodrug is rapidly converted to the active form under physiological conditions but is considerably more stable at low temperatures and pH values

The docetaxel derivative is an ester prodrug generated by attaching the weak-base derivatizing unit, N-methyl-piperazinyl butanoic acid, at the C-2′ hydroxyl group of the drug (Fig. 1); the free hydroxyl group in the C-2′ position is essential for activity [27–29]. Amino esters based on N-methyl-piperazine are commonly used for improving the water-solubility of drugs and are known to be readily removed by esterases in the body [30]. For development as a LNP formulation the prodrug has to be stable at low pH as found inside the liposomal nanocarrier and has to be rapidly converted into its active form (docetaxel) once it is released from the LNP. The following experiments evaluate the chemical/hydrolytic stability of the docetaxel derivative in aqueous buffers and plasma, a medium that closely mimics the physiological situation and determines the solubility and pK_a of the prodrug.

The prodrug was incubated in buffers of different pH values at different temperatures and prodrug and parent drug (docetaxel) levels determined as a function of time by UHPLC-UV. As expected, the half-life of the prodrug decreased dramatically at higher temperatures and pH values, ranging from 49 days at pH 4/7 °C to 7 days at pH 7.5/7 °C and 10 h at pH 7.5/37 °C. The prodrug conversion in buffered mouse plasma (pH 7.5) at 37 °C was even faster and occurred with a halftime of 1 h. These results demonstrate that the prodrug is reasonably stable at the low pH values found inside the liposomes (pH around 4) and can undergo rapid conversion into active drug following release from the LNP carrier into the blood circulation or at the disease site. This conversion is pH-dependent (faster at higher pH) and is significantly accelerated in the presence of hydrolytic enzymes present in biological fluids such as blood plasma.

The pK_a of the derivative was determined by acid-base titration and was found to be 7.7, making it well suited for pH gradient loading into LNP. The water solubility of the hydrochloride salt was 2.8 mg/ml at pH 4 and decreased with increasing pH (1.7 mg/ml at pH 5 in 10 mM acetate buffer and 0.15 mg/ml at pH 7.4 in phosphate buffered saline). The solubility at low pH is 250–500 times higher than the published solubility of the parent compound docetaxel (5–10 µg/ml).

The ability of the prodrug to form the active drug (bioconversion) was further investigated *in vitro*. The anticancer activity of the docetaxel derivative was evaluated in comparison to docetaxel in a panel of 3 human cancer cell lines (breast, prostate and ovarian cancer). Cytotoxicity of the prodrug and parent drug (docetaxel) was determined using the Alamar Blue assay after a 72-h drug exposure period. IC_{50} values (nM) are presented in Table 1. The derivative was as active as the parent drug docetaxel demonstrating that the prodrug is readily converted into active drug.

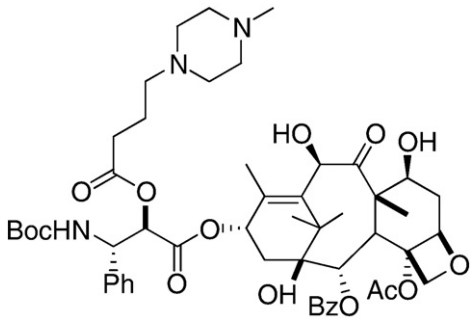

Fig. 1. Structure of 2′-O-(N-methyl-piperazinyl butanoyl) docetaxel (free base).

Table 1
In vitro cytotoxicity (IC_{50} values) of docetaxel and docetaxel derivative.

Cell line	IC_{50} (nM)	IC_{50} (nM)
	Docetaxel	Derivative
PC-3 (prostate cancer)	1	0.5
MDA-MB-435/LCC6(breast cancer)	<0.1	<0.1
ES-2 (ovarian cancer)	0.1	0.1

3.2. The docetaxel derivative can be efficiently loaded and retained in LNP

The docetaxel prodrug was loaded into LNP using the ammonium sulfate loading technique. This loading method was chosen, as it works well in acidic media where the prodrug is most stable. Trapping efficiencies approaching 100% were achieved within 15 min of incubation at 60 °C for drug-to-lipid weight ratios as high as 0.4 mg/mg (Supplementary Fig. 1). No breakdown of the prodrug was observed by UHPLC during loading when the pH was maintained at 5.5 or lower. QELS analyses of the loaded LNP indicated no significant changes in average size and size distribution (100 ± 20 nm). Optimal LNP loading was achieved by incubation in pH 5 buffer at 60 °C for 30 min. In all subsequent experiments LNP were loaded at an initial drug-to-lipid ratio of 0.2 mg/mg (5 mg/ml lipid) using the optimal loading conditions. Formulations were stable over a four months observation period under cold storage conditions (data presented below).

3.3. LNP-encapsulated docetaxel derivative exists in a crystalline form and is highly stable

The stability of the LNP docetaxel derivative formulation was monitored over a period of 4 months. DSPC/Chol, DPPC/Chol and DMPC/Chol docetaxel derivative formulations (prodrug-to-lipid ratio 0.2 wt/wt) were stored at 7 °C in sealed sterile vials; at various time intervals (once a week within the first month and monthly thereafter) LNP size, prodrug retention and prodrug integrity were analyzed. The results are summarized in Supplementary Fig. 2A–C. All three formulations were extremely stable; prodrug release was not detectable, the average size and size distribution of the LNP formulations remained unchanged and prodrug hydrolysis was less than 4% (3.5–3.8%). No other degradation products were observed.

The results presented above indicate that LNP encapsulation leads to greatly improved hydrolytic stability of the entrapped prodrug compared with that of the prodrug in aqueous solution. The intravesicular pH is approximately 4 [31]. At this pH the prodrug had a hydrolysis half-life of about 7 weeks. In contrast, less than 4% of the encapsulated prodrug was converted into docetaxel over a period of 16 weeks. It has been noted that encapsulation of drugs such as doxorubicin in LNP can result in the formation of precipitates inside the LNP [32]. Drug present in such precipitates would be expected to exhibit enhanced stability properties and could result in the increased stability of the formulated prodrug. This possibility was investigated by cryo-TEM. Cryo-TEM micrographs revealed the presence of electron dense rod-shaped structures in the interior of prodrug-loaded LNP (Fig. 2, right-hand side and inset), which were not observed for the prodrug-free vesicles (Fig. 2, left-hand side). This indicates that the prodrug is precipitated in the LNP interior in the form of linear “needle-like” structures.

Having established that the prodrug is active, can be efficiently loaded into LNP and LNP formulations are stable, *in vivo* studies were performed in mice to determine tolerability, pharmacokinetic properties and efficacy of the prodrug alone or formulated in LNP in comparison to Taxotere™, the commercial docetaxel formulation.

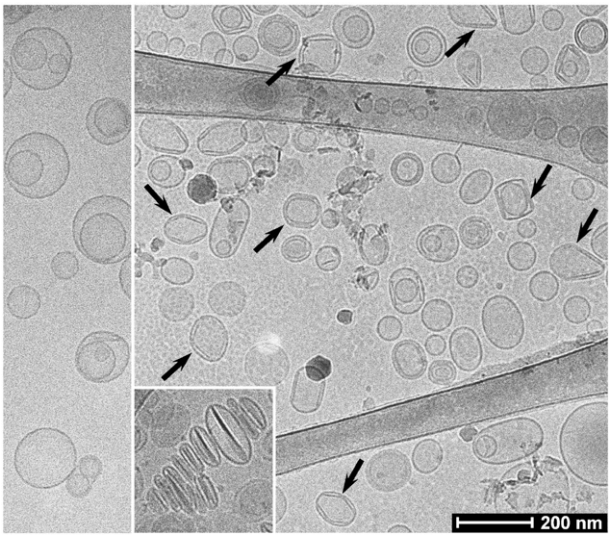

Fig. 2. Cryo-TEM images of DSPC/Chol (55/45 mol.%) LNP. (Left) LNP without drug. (Right) Loaded with docetaxel derivative at a prodrug-to-lipid weight ratio of 0.2 mg/mg. The arrows indicate drug precipitate. The inset shows LNP loaded with the doceatxel derivative at a prodrug-to-lipid weight ratio of 0.37 mg/mg. The drug forms a rod-shaped precipitate in the center of the LNP giving the LNP a coffee bean-like appearance. The bar in the micrograph represents 200 nm.

3.4. LNP formulations of the docetaxel derivative are better tolerated than docetaxel/Taxotere™

The tolerability studies were aimed at establishing the maximum tolerated dose (MTD) and the dose range for the efficacy studies (efficacy studies were based on a single i.v. injection). Single dose MTD studies were performed in immune-compromised SCID/Rag2-M mice (the same mouse strain used for the efficacy studies). The prodrug alone formulated in the same manner as docetaxel in Taxotere™ (using the surfactant polysorbate 80) and in LNP was compared to Taxotere™. Tolerability was assessed by changes in body weight as well as behavioral parameters. The MTD was defined as the dose that results in less than 15% loss in body weight and does not cause lethality. The single dose MTD of docetaxel/Taxotere™ was 29 mg/kg. In contrast, the MTD of the docetaxel prodrug was only 16 mg/kg (MTD in docetaxel equivalents corresponding to 20 mg/kg prodrug). The prodrug showed acute toxicity (lethality observed within 5 min of injection) at a docetaxel equivalent dose of 20 mg/kg. Vehicle (polysorbate 80/physiological saline) alone had no adverse effects. The LNP-encapsulated derivative (DSPC/Chol 55:45 mol% LNP, prodrug/lipid weight ratio 0.2 mg/mg) was well tolerated with no signs of toxicity (no significant changes in body weight and behavioral parameters) at docetaxel equivalent doses as high as 88 mg/kg. The MTD of the LNP formulation is at least 3 times higher than that of docetaxel/Taxotere™ (29 mg/kg) demonstrating that it is much better tolerated (less toxic) than the latter and hence can be administered at much higher more efficacious doses. The results are summarized in Table 2. Additional data including maximum weight loss data, drug-related death data and clinical observations are provided in Supplementary Table 1.

3.5. The LNP-encapsulated derivative exhibits long circulation lifetimes following i.v. administration

The next set of studies examined the rate of elimination of docetaxel/Taxotere™, the derivative formulated in polysorbate 80 and in LNP from the blood circulation of female Swiss Webster mice. Drugs were administered i.v. through a single bolus injection at equimolar doses (20 mg/kg docetaxel). Both docetaxel/Taxotere™ and the docetaxel derivative had plasma circulation half-lives of minutes

Table 2
Maximum tolerated doses (MTD) of Taxotere™, the docetaxel derivative formulated in the same manner as Taxotere™ and encapsulated in DSPC/Chol (55:45 mol%) LNP at a prodrug-to-lipid ratio of 0.2 wt/wt based on a single bolus i.v. injection.

Drug formulation	MTD (mg/kg)
Taxotere	29
LNP formulation	>88
Derivative	16

and plasma concentrations were below detectable levels by 2 h (Fig. 3). In contrast, formulation of the derivative in DSPC/Chol LNP extended the circulation half-life from minutes to about 10 h with 50–100 times higher plasma concentrations compared to docetaxel/Taxotere™. Approximately 24% of the injected dose remained in the circulation at 16 h. The elimination of the LNP-formulated derivative appears to be primarily determined by the elimination rate of the LNP carrier. These data demonstrate that LNP formulations of the derivative are stable in circulation and can achieve circulation half-lives that favor efficient drug accumulation at tumor sites.

3.6. The rate of prodrug release from LNP can be regulated by adjusting the lipid composition

The activity of LNP-based drugs is highly dependent on the release rate of the drug from the carrier. For example, if the drug leaks out of the LNP carrier too rapidly, it will all leak out before getting to the disease site and no therapeutic benefit over free drug will be seen. The main determinants of drug retention/release are the intra-vesicular form of the drug and the lipid composition of the LNP carrier. The use of unsaturated lipids or lipids with shorter acyl chains favors faster drug release. The following studies examine the effect of lipid composition (acyl chain length) on *in vivo* drug release and correlate these data with release data obtained *in vitro*. The docetaxel derivative was encapsulated in DSPC/chol, DPPC/Chol and DMPC/Chol LNP (55:45 mol%) containing trace amounts of the lipid marker [^{3}H]CHE. Drug retention was determined by comparison of the initial prodrug-to-lipid ratio with the prodrug-to-lipid ratios found at different time points in the plasma of female Swiss Webster mice. The prodrug retention profiles *in vivo* are shown in Fig. 4. Both DSPC/Chol and DPPC/Chol LNP show no or little release over the 16 h time course of the experiment. DMPC/Chol LNP released the prodrug with a halftime of about 6 h with 16% of the prodrug remaining entrapped 16 h post injection. Comparison of the retention profiles of DSPC/Chol LNP formulations loaded at 0.1 and 0.2 mg/mg showed similar release rates (data not shown). *In vitro* release studies performed in mouse plasma show the same trend as the *in vivo* studies (data not shown). The increase in release seen with DMPC/Chol LNP compared to DSPC and DPPC/Chol LNP is consistent with a decrease in membrane permeability in going from DMPC, which has the shortest acyl chains (C_{14}) to the longer chain lipids (C_{16} and C_{18}). The data demonstrate that the prodrug can be efficiently retained in LNP and the release rates can be regulated by varying the lipid composition of LNP carrier.

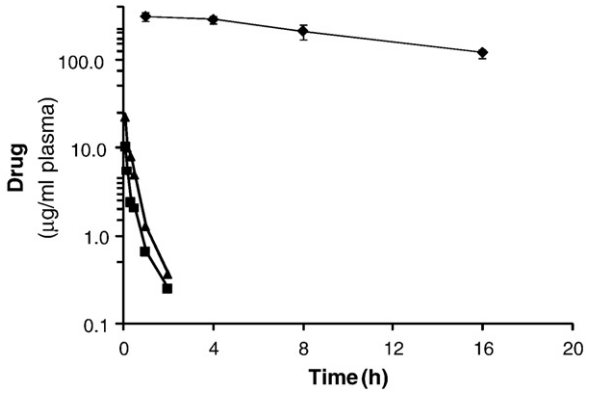

Fig. 3. Plasma elimination profiles of Taxotere™ (▲), docetaxel derivative formulated in the same manner as Taxotere™ (ethanol/polysorbate 80/physiological saline) (■) and DSPC/Chol LNP formulation of the derivative (prodrug-to-lipid ratio 0.2 (wt/wt)) (♦) following i.v. administration in mice. Female Swiss Webster mice were injected intravenously with a single dose of the various formulations at equimolar doses (20 mg/kg docetaxel). Prodrug levels in plasma were determined by UHPLC-MS. Data points represent mean values ± standard deviation from each group of mice ($n=4$).

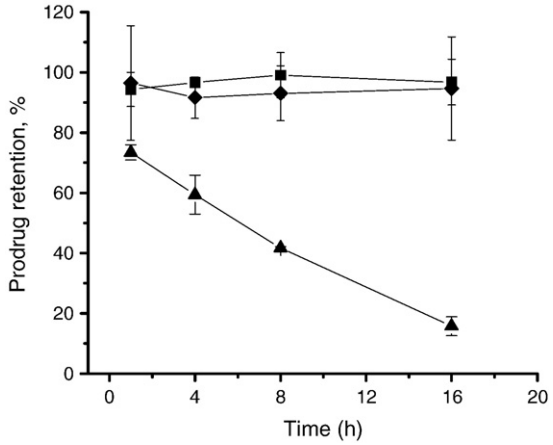

Fig. 4. Prodrug retention in DSPC/Chol (♦), DPPC/Chol (■) and DMPC/Chol (▲) LNP formulations determined *in vivo*. LNP formulations containing trace amounts of the radiolabeled lipid [^{3}H]-CHE were injected intravenously into female Swiss Webster mice at a docetaxel equivalent dose of 20 mg/kg. Plasma samples taken at the indicated time points were analyzed for lipid and prodrug content by liquid scintillation counting and UHPLC, respectively. Each data point represents mean values ± standard deviation from each group of mice ($n=4$).

3.7. LNP-encapsulated docetaxel derivative can be more potent and less toxic than docetaxel/Taxotere™ in a breast cancer model

The anticancer efficacy of different LNP-prodrug formulations was evaluated in a subcutaneous xenograft model of human breast cancer (MDA-MB-435/LCC6) after a single bolus injection. The first set of studies assessed the effect of lipid composition/drug release rates on activity using the same formulations as for the release studies (DSPC/Chol, DPPC/Chol and DMPC/Chol). The second set of studies compared the most active LNP formulation at equimolar and equitoxic doses with Taxotere™. The influence of lipid composition of the LNP carrier on the efficacy of encapsulated prodrug is illustrated in Fig. 5A. DSPC/Chol, DPPC/Chol and DMPC/Chol LNP formulations (prodrug-to-lipid ratio 0.2 wt/wt) were administered at a docetaxel equivalent dose of 40 mg/kg. The DSPC/Chol formulation inhibited the tumor growth most effectively, followed by the DPPC/Chol and DMPC/Chol (least active) formulations. The antitumor efficacy indicates that the formulation that exhibits the slowest drug release is most active (Fig. 5A, inset). The therapeutic activity of the DSPC/Chol formulation (prodrug-to-lipid ratio 0.2 wt/wt) was determined at 3 different doses (25, 40 and 88 mg/kg docetaxel) in comparison to 25 mg/kg Taxotere™/docetaxel. At equimolar doses (25 mg/kg docetaxel) the LNP formulation and Taxotere™ had similar activity (not significantly different, $p>0.05$). However, due to the fact that it is well tolerated, the LNP formulations can be administered at doses much higher than the MTD of Taxotere™. At dose levels corresponding to 88 mg/kg docetaxel (a dose level that is still well below the MTD of LNP docetaxel derivative) the DSPC/Chol LNP formulation was much more efficacious ($p<0.001$, Fig. 5B). The most significant tumor growth suppression was observed at 88 mg/kg docetaxel with a tumor growth delay (T-C; time for treatment (T) and control groups (C) to double relative tumor size from 1 to 2) of 29 days compared to 9 days

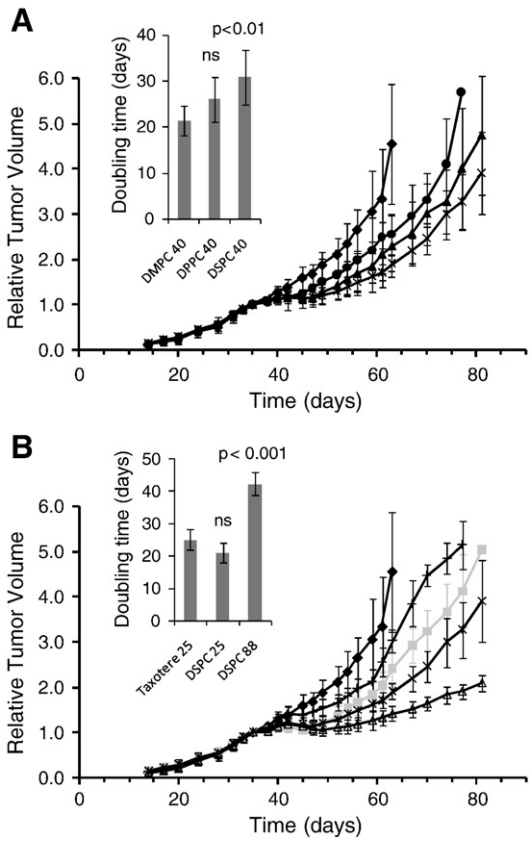

Fig. 5. Anticancer efficacy. Response of subcutaneous MDA435/LCC6 human breast carcinoma xenografts to treatment with Taxotere™ and LNP-encapsulated docetaxel derivative in Rag2M mice. (A) Treatment with various LNP formulations to determine the effect of lipid composition on efficacy. LNP formulations (prodrug-to-lipid ratio 0.2 wt/wt) were composed of DSPC/Chol (x), DPPC/Chol (▲) and DMPC/Chol (●) and administered at a docetaxel equivalent dose of 40 mg/kg. Untreated control received a saline injection (♦). The inset compares the DMPC/chol LNP formulation with the DPPC/chol and DSPC/chol formulations in terms of tumor size doubling times (time it takes tumors to increase two-fold in relative size (from 1 to 2)); ns stands for not significantly different. (B) Dose-response for the DSPC/Chol LNP formulation (prodrug-to-lipid ratio 0.2 wt/wt) and comparison to Taxotere™. The formulation was administered at docetaxel equivalent doses of 25 (+), 40 (x) and 88 (Δ) mg/kg. Untreated control received a saline injection (♦). Taxotere™ (■) was admistered at 25 mg/kg. The inset compares Taxotere with the DSPC/chol formulations in terms of tumor size doubling times. Tumor growth curves are shown with standard deviations. Treatment was initiated at day 35 with a single i.v. bolus injection. Points represent the means of relative tumor volumes (ratio of the tumor volume measured at a given time point to the tumor volume measured at the treatment day), mean values for 6 mice per group are presented.

for Taxotere™ dosed at its MTD (Supplementary Table 2). These results demonstrate that LNP formulations can be much more effective than Taxotere™.

4. Discussion

The results presented in this study indicate that a weak base prodrug derivative of docetaxel can be effectively loaded into LNP systems, resulting in formulations that are considerably less toxic than docetaxel and that can exhibit enhanced efficacy compared to free docetaxel/Taxotere™. There are two aspects of these results that warrant further discussion. The first concerns the properties of the LNP formulation in comparison with other formulations of docetaxel in the clinic or in clinical development and whether LNP docetaxel derivative would be expected to exhibit superior therapeutic properties. Second, it should be emphasized that the approach detailed here is a general methodology that can be applied not only to established drugs such as docetaxel but also to new chemical entities (NCEs) that may not exhibit properties consistent with administration in the "free" form.

The LNP formulation of the docetaxel derivative represents the first LNP formulation of a taxane where the drug is actively loaded into the aqueous core of the LNP and stably retained within the carrier. Previously reported LNP formulations of a taxane-based drug (paclitaxel) have shown no major differences in plasma elimination rates between free drug and its liposomal form [17,18]. In the present work, we observed notably prolonged circulation for LNP-formulated prodrug compared to its free form and the parent compound. In previous LNP taxane formulations the LNP serve primarily as excipients, the taxane escapes from the lipid carrier immediately after injection. It should be noted that the excipients used for paclitaxel (Cremophor) and docetaxel (Tween 80) can cause hypersensitivity reactions [8–13] that are typically not observed for LNP formulations. The LNP formulation of the docetaxel derivative clearly offers an additional major advantage in that the drug is retained in the carrier, which can lead to enhanced tumor accumulation of LNP-encapsulated prodrug through the enhanced permeability and retention mechanism (EPR effect) utilizing the vascular abnormalities of solid tumors [33–36]. In addition it may be expected that the LNP docetaxel derivative will exhibit more uniform PK characteristics compared to the Cremophor and Tween 80 formulations of paclitaxel and docetaxel respectively. It may be noted that at a drug-to-lipid ratio of 0.2 (wt/wt) a dose of 100 mg/kg of the docetaxel derivative corresponds to a lipid dose of 500 mg/kg, well above the dose level required for uniform, long circulation lifetimes for LNP carriers [37].

The results presented here also indicate that LNP encapsulation greatly reduces the toxicity of the prodrug. While the derivatized docetaxel formulated in the surfactant polysorbate 80 exhibited an MTD lower than that of parent compound, LNP encapsulation of the prodrug resulted in dramatically reduced toxicity as indicated by survival and weight loss data. The increase in MTD (>88 mg/kg docetaxel equivalents) for LNP-formulated prodrug could be due to reduced systemic exposure to drug (resulting from the decreased volume of distribution and lower exposure of normal tissues) as well as reduced vehicle toxicities (non-toxic LNP lipids vs. polysorbate 80). The non-toxic nature of the LNP formulations of derivatized docetaxel allows more drug to be administered and greater efficacy to be achieved. It should be noted that the LNP formulation also allows sustained release of the prodrug at the tumour site which can lead to enhanced efficacy [38] for cell-cycle specific anticancer drugs.

The enormous clinical utility of the taxanes, and the limitations of the surfactant-based formulations [8–13], have resulted in considerable efforts to generate improved formulations. To date the most successful approaches involve the use of drug delivery systems. Delivery systems can be less toxic than the surfactants and have the potential to reduce systemic drug exposure and increase drug delivery to tumors. Approaches that have resulted in approved formulations or are currently undergoing clinical testing can be divided into three main categories including: (1) protein-based carriers (albumin nanoparticles, Abraxane, Abraxis); (2) liposomes (LEP-ETU and LE-DT, Neopharm), emulsions (Tocosol, Sonus) and polymer micelles (Genexol-PM, Samyang Corp.); and (3) macromolecular drug conjugates (Xyotax, Cell Therapeutics) [9,14–18,39–48]. These formulation approaches with the exception of LE-DT (liposomal docetaxel) have focused on paclitaxel. Abraxane was approved in 2005; LEP-ETU, Tocosol, Genexol-PM and Xyotax are currently in clinical phase II and III trials and LE-DT is in clinical phase I [48]. These formulations offer some advantages over the older established taxanes, mostly in ease of administration and reduced overall toxicity, related to the absence of Cremophor and Tween 80 (no pre-medication required, shorter infusion times), which in some instances allows the administration of higher doses. However, the improvements in effectiveness are only modest, if any [9]. The reasons for the limited activities of the

formulations summarized above become obvious when the nature of association of the taxanes with the carrier is considered. Hydrophobic interactions are responsible for the association of the drug with the protein (category 1) and amphiphile-based (category 2) delivery systems. These interactions can be relatively weak, leading to rapid dissociation of the drug from the carrier and a reduction of the amount of drug that can reach the tumor through extensive drug distribution. In the case of macromolecular drug conjugates (category 3), the association of the drug can be too stable, as the drug is covalently bound to a hydrophilic polymer and activity requires degradation of the polymer matrix to liberate the active drug. A comprehensive review can be found in Hennenfent and Govindan [9]. In summary, these formulations show that both the lack of stable association of the drug with the carrier (drug is removed rapidly from circulation and no advantage over standard taxane therapy is observed), as well as too stable association (drug is not bioavailable), can reduce anticancer activity. More recently, mainly due to Taxotere™ coming off patent in 2010 research efforts have focused on the development of alternative docetaxel formulations. These formulations are at the preclinical development stage [49–51].

The approach used here to load docetaxel into LNP by converting it to a weak-base prodrug has broad applicability. Many potentially effective chemotherapeutic agents identified at early stages of drug discovery have problems associated with solubility and bioavailability that lead to poor penetration to diseased tissue in vivo resulting in compromised efficacy and toxic side effects. Medicinal chemists traditionally approach this problem by modifying drug compounds to achieve improved pharmacokinetic and bioavailability properties. However, the approach outlined here indicates that modifications leading to an ability to load the compound in LNP can solve many of the issues required to make a bioactive agent an effective drug. In the present work, the therapeutic utility of this approach has been demonstrated with the successful design of a LNP formulation of the anticancer agent docetaxel, a hydrophobic, poorly water-soluble drug that cannot normally be stably encapsulated and retained in LNP. Stable association of this drug with the LNP carrier was achieved through a chemical modification of the drug to form a weak base derivative that can be actively loaded into LNP using pH-gradient loading techniques.

The derivatization of docetaxel was performed by attaching the amine-containing carboxylic acid, N-methyl-piperazinyl propionic acid, to the reactive C-2′ hydroxyl group [27]. Piperazine groups are common structural motifs in many drugs such as the quinolone-based antibacterials ciprofloxacin and norfloxavin [52]; the N-methyl-piperazino modifying group has been used in developing the anticancer agent Glivec [30]. As shown here, derivatization of docetaxel with an N-methyl-piperazino group not only resulted in the formation of a protonable, water-soluble prodrug, but also allowed to achieve a desirable balance of the hydrolytic stability/lability that is necessary to render the active drug bioavailable. While the LNP encapsulated prodrug has to remain stable inside the LNP carrier, it should be activated *in situ* under physiological conditions following release from the LNP vehicle. The prodrug exhibited favorable properties in this regard and multiple mechanisms can contribute to activation under physiological conditions including pH and the presence of hydrolytic enzymes. The prodrug was relatively stable at pH 4 (the pH inside the LNP) and progressively converted into the active drug as the pH was raised (10 h half-life at pH 7.4). Although the prodrug is readily converted into active drug at physiological pH (pH 7.4) the rapid conversion in mouse plasma (1 h half-life) points to a major role of esterases in this process.

As demonstrated further, the use of the ammonium sulfate-based remote loading method resulted in efficient and stable entrapment of the derivative in the 100 nm LNP. This is reflected by trapping efficiencies approaching 100% at drug-to-lipid ratios as high as 0.4 wt/wt. The prodrug was released only slowly from the LNP formulations in the blood circulation and exhibited increased chemical stability compared to prodrug in bulk aqueous solution. Enhanced stability and slower release rates have been observed for a number of liposomal drugs that tend to form insoluble precipitates or gels inside the LNP [32,38,53,54]. Formation of an intravesicular precipitate was confirmed by cryo-TEM (Fig. 2).

We believe that the ability of the technology presented here to efficiently retain the prodrug and release it at a controlled rate at the tumor site could have a significant impact on the effectiveness of anticancer agents by increasing both the intensity and the duration of tumor drug exposure. This may lead to a number of specific advantages. First, LNP encapsulation of otherwise non-loadable drugs may result in superior anticancer activity with fewer side effects compared to standard clinical formulations. Second, accelerated clinical development at lower cost can be expected due to the use of a well-characterized drug class with proven clinical utility. Third, ease of development due to straightforward chemical derivatization procedures, use of established amine groups/linkers, and well established readily scaleable LNP technology. Finally, this approach can be considered as not only limited to anticancer drugs, but representing a platform technology that can be applied to other approved therapeutic agents, as well as to new chemical entities that are specifically designed for use in a LNP carrier.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jconrel.2010.02.029.

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