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ORIGINAL ARTICLE

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Liposome formulation of a novel hydrophobic aryl-imidazole compound for anti-cancer therapy

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Abstract *Purpose*: A cholesterol-free liposome formulation formed from mixtures of egg phosphatidylcholine (ePC) and poly (ethylene glycol) conjugated distearoylphosphatidylethanolamine (DSPE-PEG 2000) was optimized and evaluated for delivery of a novel anti-cancer agent ML220 (2-(5-bromo-1H-indol-3-yl)-1H-phenanthro [9,10-d] imidazole). *Results and Discussion*: ML220 is highly lipophilic with a water solubility of 0.14 μg/ml and calculated log *P* of 5.69. The ML220-loaded liposomes had a unimodal size-distribution and a mean diameter of 89 nm. The drug to lipid ratio in the formulation was 1:3.5 (mol:mol) and the drug loading efficiency was 83% providing a more than 50,000-fold increase in the water solubility of ML220. The formulation was demonstrated to be stable in vitro at 37°C for over 2 weeks with a delayed drug release profile. Evaluation of the subacute toxicity of the liposome formulated drug in C3H mice revealed no overt signs of toxicity. Also, a biexponential drug plasma concentration pattern was found upon evaluation of the pharmacokinetics in Balb/C mice. The in vivo evaluation of the anti-cancer activity in a human colon HT29 carcinoma model revealed a significant delay in tumor growth. *Conclusion*: Overall, the ePC/DSPE-PEG liposomes were demonstrated to be a suitable delivery system for ML220. These studies also highlight the potential of cholesterol-free liposomes as a formulation strategy for highly lipophilic drugs.

Keywords Cholesterol-free liposome · Hydrophobic drug · Anti-cancer · Drug delivery · Solubilization · Aryl-imidazole

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Introduction

Efforts in medicinal and combinatorial chemistry continue to give rise to a wide range of anti-cancer agents with great therapeutic potential. However, many of these agents have solubility, stability or toxicity issues that retard or prevent their development into viable treatment strategies. In many cases, delivery or formulation technologies are investigated as a means to exploit the therapeutic potential of these agents. To date, many nano-sized systems such as liposomes [1, 2], micelles [3, 4], and nanoparticles [5] have been explored for systemic delivery of anti-cancer agents. Among these technologies, liposomes have become the most well characterized and well established [2, 6–8]. Liposomes have been explored for the solubilization and delivery of hydrophilic (e.g., cytarabine, log $P = -1.25$), hydrophobic (e.g., paclitaxel, log $P = 3.96$) and amphiphilic compounds (e.g., doxorubicin, log $P = 0.17$; daunorubicin, log $P = 0.73$) [6, 9, 10]. They have been proven to be particularly effective in the delivery of amphiphilic molecules that are weak acids or weak bases due to the inherent sensitivity of these molecules to active loading techniques [6, 11] Indeed, there are FDA approved formulations relying on liposome technology for the amphiphilic compounds doxorubicin (Doxil®, Myocet®) and daunorubicin (DaunoXome®).

Amphiphilic and hydrophilic compounds are entrapped within the aqueous interior of the liposomes; while, hydrophobic drugs intercalate within the hydrophobic region of the lipid bilayer [6]. In this way, for effective formulation of hydrophobic drugs, the bilayer must provide a suitable microenvironment for solubilization of the drug. Recently, cholesterol containing liposomes have been explored quite extensively for formulation of the hydrophobic anti-cancer agent paclitaxel [12–15]. A liposomal formulation of paclitaxel (LEP-ETU, NeoPharm Inc.) is currently in phase I clinical trial evaluation [14]. To date, the loading efficiencies for stable liposomal formulations of this

hydrophobic drug are typically ≥90%. However, the maximum drug to lipid ratios achieved for these liposomal formulations of paclitaxel have been equal to or less than 1:33 (mol:mol) [14]. The low solubilization or drug loading capacity of these formulations may be attributed to the presence of cholesterol. Recently, Zhang et al. [16] reported that an increase in the cholesterol content (i.e., 5–37 mol%) within the composition of the liposomes resulted in a significant decrease in the loading efficiency for paclitaxel (i.e. 99–6%). Cholesterol is a bulky, robust molecule that intercalates within the hydrophobic space of the lipid bilayer. Therefore, the presence of cholesterol may reduce the hydrophobic space available for incorporation of hydrophobic drugs [16–18]. To date, very few studies have explored the use of cholesterol-free liposomes as a formulation strategy for hydrophobic drugs.

In the present study, cholesterol-free liposomes formed from mixtures of ePC and DSPE-PEG 2000 were used to formulate ML220, the new anticancer agent. ML220 is an aryl-imidazole (2-(5-bromo-1H-indol-3-yl)-1H-phenanthro[9,10-d]imidazole) that has been demonstrated to have potent anti-proliferative activity against a variety of human cancer cell types [19, 20]. The main anti-cancer mechanism for this agent has been identified to include the induction of a partial arrest in the G_0/G_1 phase of the cell cycle. Specifically, ML220 has been found to be internalized rapidly into treated cancer cells and then detected in the perinuclear area of the cells closely associated with the endoplasmic reticulum [19, 20]. ML220 has also been shown to be the inhibitor of kinase enzymes that are involved in many of the cell-signaling pathways. The subcellular distribution and morphological changes induced by ML220, as well as its selective pattern of kinase inhibition indicate that this compound targets novel mechanisms of signal transduction which, in turn, supports its promising potential as a novel anti-cancer therapy agent.

However, the pre-clinical evaluation of this compound has been limited due to its low water solubility and high protein binding affinity. In the current study, the lipid formulations of ML220 were prepared by the high pressure extrusion method and characterized in terms of size, size distribution, loading capacity, loading efficiency, stability and in vitro release kinetics. Based on consideration of the results from the physicochemical characterization of the formulations, the optimal composition (i.e. ratio of ePC:DSPE-PEG) was selected for further evaluation in vitro and in vivo. The in vitro cytotoxicity of the free and lipid formulated drug were examined in two human cancer cell lines. The pharmacokinetics profile and biodistribution of both the drug and lipid were measured following intravenous (i.v.) administration of the formulation in Balb/C mice. Finally, the in vivo subacute toxicity of the lipid formulated ML220 was evaluated in C3H mice and the anti-tumor efficacy was tested in a model of human colon adenocarcinoma in nude CD-1 mice. Overall, the cholesterol-free liposome formulation increased the aqueous solubility of ML220 by more than 50,000-fold and was found to have promising therapeutic potential.

Materials and methods

Materials

Phosphatidylcholine from egg yolk (ePC, purity = 98.8% PC) and DSPE-PEG 2000 (distearoylphosphatidylethanolamine covalently linked to polyethylene glycol with molecular weight 2,000) were obtained from Northern lipids Inc. (Vancouver, BC, Canada). Dialysis membrane (MWCO = 1,000) was purchased from Spectrum Laboratories Inc. (Dominguez, CA) and the ^{3}H-labeled 1,2-Dipalmitoyl-sn-Glycero-3-Phosphocholine (DPPC) was obtained from Perkin Elmer Inc. (Woodbridge, ON, Canada). ML220 was supplied by Lorus Therapeutics Inc (Toronto, ON, Canada). All other chemicals used in this study were HPLC or analytical grade and purchased from Aldrich Inc. (St. Louis, MO, USA).

Characterization of ML220

Measurement of aqueous solubility of ML220 in the absence and presence of physiologically relevant concentrations of albumin

Double distilled water or buffer with 45 g/l bovine serum albumin (BSA) was saturated with ML220 by the addition of drug as dry powder until obvious precipitation was observed. The solutions were vortexed, left to stir for 24 h at room temperature and then centrifuged for 30 min at 14,000 rpm (Eppendorf 5804R, Eppendorf Inc., Hamburg, Germany). An aliquot of supernatant was then carefully removed and centrifuged again as previously described in order to ensure complete removal of the precipitated drug. An aliquot of supernatant was then removed, dried under nitrogen and re-suspended in DMF. The concentration of ML220 was determined using the UV assay described in detail in "Measurement of drug loading efficiency".

Calculation of log P

The log *P* value for ML220 was calculated using CAChe WorkSystem Pro Version 6.1.10 software (Fujitsu Inc. Beaverton, OR, USA).

Preparation of liposome formulations of ML220

Stock solutions of ML220 and lipid (ePC and DSPE-PEG) were prepared in acetone and chloroform, respectively. Specific volumes of the stock solutions of drug and lipid were then mixed in order to achieve a

final lipid concentration of 25 mg/ml [ePC : DSPE-PEG = 95:5, 90:10, 80:20 (mol%)] and a drug to total lipid ratio of 1:10 (w/w). The mixtures were stirred for 4 h, thoroughly dried under nitrogen and left overnight under vacuum. Hepes buffer saline (HBS 0.01 M, pH = 7.4) warmed to 60°C, was then added in order to rehydrate the dried films. The solutions were vortexed, stirred for 48 h at room temperature and sonicated for one and half hours prior to being extruded ten times in a 10 ml thermobarrel extruder (Northern Lipids, Vancouver, BC, Canada) fit with a single 0.1 μm polycarbonate membrane (Whatman, Clifton, NJ, USA). After extrusion, all solutions were lyophilized to powder and re-suspended in HBS (0.01 M, pH = 7.4) prior to use.

For the pharmacokinetics and tissue distribution studies, ^{3}H-labeled DPPC (i.e., one of the main components of ePC) was added to the formulation as a tracer. The ^{3}H-labeled liposomes were prepared as outlined above. The final concentration of ^{3}H-labeled DPPC in the formulation was 7×10^{-6} mg/ml.

Determination of liposome size and morphology

The hydrodynamic diameter of liposomes was measured by dynamic light scattering (DLS) (Dynapro DLS, Protein Solutions, Lakewood, NJ, USA). For the analysis, the samples were diluted in filtered double distilled water. The morphology of the vesicles was assessed using both multi-angle light scattering and transmission electron microscopy (TEM).

Transmission electron microscopy

The TEM analysis of the liposomes was performed using a Hitachi HD-2000 microscope operating at an acceleration voltage of 200 kV. The liposome solutions were diluted in HBS buffer and deposited on copper grids that had been pre-coated with carbon and negatively charged.

Multi-angle dynamic light scattering

Light-scattering measurements were carried out with an ALV SLS/DLS 5000 goniometer/spectrometer (ALV GmbH, Langen, Germany). The light source employed was a JDS Uniphase He–Ne laser ($\lambda_0 = 632.8$ nm, 35 mW) emitting vertically polarized light. For analysis, the samples were diluted in filtered double distilled water. The light scattering signal was collected at angles ranging from 30° to 150° (at 10° intervals).

Thermal analysis

Thermal analysis was performed on 10 μl aliquots (i.e., 10 mg) of the liposome sample using a Q100 differential scanning calorimeter (DSC) (TA Instruments, Inc., New Castle, DE, USA). The samples were cooled to −20°C using a refrigerated cooling system and then heated to 10°C at a rate of 0.5°C/min. The data was analyzed using TA universal software (TA Instruments, Inc., New Castle, DE, USA).

Measurement of drug loading efficiency

The amount of ML220 incorporated into liposomes was determined using a method based on detection by UV absorbance at a wavelength of 330 nm (Perkin Elmer Corp. Lambda2, Norwalk, CT). In short, 20 μl of each liposome solution was diluted in DMF (1:1,000 v/v) and vortexed in order to disrupt the liposomes. The drug loading efficiency (DLE) was calculated using the following equation:

$$\text{DLE}\ (\%\ ) = \frac{\text{amount of drug in liposome}}{\text{amount of drug initially added}} \times 100.$$

Evaluation of in vitro stability of liposomes

The stability of the lipid formulations of ML220 following re-suspension in HBS was evaluated in both the absence and presence of physiologically relevant concentrations of BSA. The ML220-loaded liposome solutions were mixed with equal volumes of either a solution of BSA (45 g BSA/l in 0.01 M HBS) or HBS (0.01 M) and incubated at 37°C. At various time points, 50 μl aliquots were removed and analyzed by DLS ($n = 3$). The DLS analysis of samples was performed as outlined above ("Determination of liposome size and morphology").

Measurement of in vitro drug release of ML220 from liposomes

The liposome solutions used for the release studies were prepared by re-suspending the lyophilized formulations in HBS buffer to achieve a final lipid concentration of 100 mg/ml and drug concentration of 8.3 mg/ml. The in vitro release of ML220 from the liposomes was evaluated using the dialysis method [21]. In short, the solution containing the drug was placed in a dialysis bag that was suspended in 0.5 l of HBS (0.01 M pH = 7.4) at 37°C. At given time points, 20 μl aliquots were withdrawn from the dialysis bag for analysis. The volume of the solution in the dialysis bag was measured at each time point. At 1, 24, 72 and 168 h the external medium was removed and replaced with fresh buffer in order to ensure sink conditions were maintained. The concentration of ML220 in the withdrawn solution was measured by a UV-based method as outlined above.

Evaluation of in vitro cytotoxicity of ML220 formulated in liposomes

The in vitro cytotoxicity of ML220-loaded liposomes and free ML220 were evaluated in the human ovarian carcinoma cell line SKOV-3 and the human breast carcinoma cell line MCF-7. SKOV-3 and MCF-7 cells were maintained in RPMI 1640 medium and DMEM medium supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) penicillin–streptomycin (100 U/ml penicillin G and 100 μg/ml streptomycin), respectively. Cells were allowed to grow in a monolayer in a flask incubated at 37°C in 5% CO_2 and 90% relative humidity. The cells were seeded in 96 well plates with a cell density of 5,000 cells/well. Following a 24-h incubation period the growth media was removed and replaced with 150 μl of fresh media containing the appropriate amounts of either free ML220 or ML220-loaded liposomes (with $n = 3$). The cells were incubated for a further 24, 48 and 72 h and the cell viability was determined using the Celltiter 96® proliferation assay (Promega Inc., Madison, WI). Specifically, 20 μl of the working reagent (i.e., 20:1 (v/v) mixture of MTS (3-(4,5)-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inert salt) and PMS (phenazine methosulfate)) was added to each well and the samples were incubated for 4 h. Cell viability was determined by measurement of the optical absorbance at $\lambda = 490$ nm using a SPECTRA MAX plus microplate reader (Molecular devices, Sunnyvale, CA, USA). For calculation of the IC_{50} (i.e., concentration of drug that provides a 50% inhibition of cell growth), the cells incubated with media alone (i.e., no treatment) were considered to have 100% viability. The IC_{50} values were extrapolated from linear regression analysis of the treatment groups.

Evaluation of in vivo subacute toxicity of the liposome formulation of ML220

The subacute toxicity of the liposome formulation of ML220 was evaluated in female C3H mice (6–7 weeks). The protocol for these studies was in accordance with the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care). The mice were divided into groups (i.e., $n = 5$ mice per group) with each receiving daily i.v. injections of 25, 50 or 100 mg/kg of ML220 formulated in liposomes over a 3-week period. As a control, one group received a daily injection of empty liposomes (i.e., no drug). The general health of the animals were assessed daily by the evaluation of appetite, level of activity and fur color. The body weights of mice were recorded on days 0, 3, 7, 10, 14, 17 and 21. Following the 3-week period, the mice were sacrificed by cervical dislocation; the liver and spleen were surgically removed and weighed as an indicator of systemic toxicity.

Evaluation of the pharmacokinetics and tissue distribution of ML220 administered in liposomes

Studies were performed using female Balb/C mice of 8–10 weeks of age. The mice were housed five per cage under standard conditions with access to food and water ad libitum. All protocols for these studies were in accordance with the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care) and were approved by the Animal Care Committee, University of Toronto, Canada. The liposome formulation of ML220 (^{3}H-labeled) was i.v.-injected into the mice via the lateral tail vein. The total ML220 dose was 75 mg/kg with an injection volume of 150 μl. ^{3}H-DPPC was selected as a marker for these studies since it is the main component of ePC. The ^{3}H-DPPC was demonstrated to be an appropriate marker for the liposomes as it was found that following administration of ^{3}H-DPPC alone (1,000,000 dpm/mouse) in mice, more than 90% of this lipid was eliminated within 2 h (data not shown). At 15 min, 1, 3, 6, 9 and 24 h post-injection, the animals were sacrificed ($n = 3$) by cervical dislocation and blood as well as tissue samples were collected (organs include heart, lung, kidney, liver and spleen). The blood samples were immediately centrifuged at 2,500 rpm for 10 min to obtain the plasma. The concentration of drug in plasma and tissue samples were determined by a HPLC method that is outlined in detail below; while, the level of lipid in plasma and tissue was measured by liquid scintillation counting.

Plasma and tissue concentrations of ML220 were determined by reverse-phase high performance liquid chromatography (Perkin-Elmer Series 200 Liquid Chromatograph with 785A UV/VIS detector). Chromatographic separations were achieved using an XTerra reverse phase column (Waters Inc., Milford, MA) with acetonitrile/methanol/water (40/40/20 v/v%) as the mobile phase and UV detection of ML220 at $\lambda = 230$ nm. All solvents were HPLC grade and filtered prior to use. For preparation of the plasma samples, the aliquots of plasma (100 μl) were mixed with 4 ml of ethyl acetate containing 0.25 μg/ml triphenylimidazole as the internal standard. The samples were then centrifuged at 5,000 rpm for 15 min, and the organic phase was transferred to a clean vial. The solvent was removed by evaporation under nitrogen and the dried film was reconstituted in 100 μl of mobile phase for HPLC analysis. For preparation of the tissue samples, the heart, lung, kidney, liver and spleen were washed to remove excess blood and then weighed. The tissue samples were then placed in excess HBS [tissue : HBS = 1:3 (w/w)], homogenized and processed as described for the plasma samples.

The precision of the HPLC method was investigated by assaying five separate samples per day for intra-day variation and three samples per day over a 3 day period for inter-day variation. The results demonstrated an acceptable repeatability with an intra-day precision of 1.8% (R.S.D.) and inter-day precision of 3.4% (R.S.D.).

The accuracy of the method was assessed using the standard addition method [22]. Briefly, samples of ML220 were prepared over a concentration range of 0.5–50 μg/ml and spiked with 0.25 μg/mL triphenylimidazole as the internal standard. The data revealed 92.3 and 89.5% recovery and R.S.D% of 2.4 and 3.5% for plasma and tissue samples, respectively.

The concentration of lipid in plasma was determined by direct scintillation counting of aliquots of the plasma samples (20 μl) in 5.0 ml scintillation cocktail (Ready Safe™, Beckman Coulter, Fullerton, CA, USA).

The total area under the blood concentration-time curves (AUC) for the drug and the lipid were calculated by the linear trapezoidal rule.

Evaluation of in vivo anti-tumor activity of ML220 administered in liposomes

Groups of five CD-1 female nude mice (6–7 weeks) were injected subcutaneously in the lower mid back with HT-29 human colon adenocarcinoma cells (3×10^6 cells in 0.1 ml PBS) and the treatment was initiated 5 days post-inoculation (size of tumors = 50–200 mm^3). The animals were treated with a daily i.v. injection of ML220 formulated in liposomes (ePC:DSPE-PEG 80:20, mol%; drug:lipid ratio of 1:3.5, mol:mol) at a dose of 75 mg ML220/kg/day for 16 days ($n = 5$). For the control group ($n = 5$), the animals were administered with sterile HBS (0.01 M, pH = 7.4). Tumor sizes were measured during the course of treatment (size of tumors = $LW^2/2$, where L and W are the major and minor axes, respectively, of the tumor measured by a caliper.). The mice were then sacrificed by cervical dislocation and the tumors were surgically removed and weighed.

Statistical analyses

The statistical analyses of the data were performed using the student's t test. Values of $P < 0.05$ were considered to be statistically significant.

Table 1 The drug loading efficiencies and drug loading levels achieved for lipid formulations that vary in terms of the total lipid concentration and the drug to lipid ratio

Lipid concentration	Drug:Lipid ratio	Loading efficiency (SD)	Loading capacity (mg/ml)
25 mg/ml	1:5	35% (4%)	1.8
	1:7.5	55% (17%)	1.8
	1:10	81% (1%)	2.0
50 mg/ml	1:5	23% (0.1%)	2.2
	1:7.5	48% (5%)	3.2
	1:10	49% (4%)	2.5

Results and discussion

Characterization of ML220 and liposome formulations of ML220

The compound of interest in these studies, ML220 (2-(5-bromo-1H-indol-3-yl)-1H-phenanthro[9, 10-d]imidazole), is highly hydrophobic, has a calculated log P value of 5.69 and a water solubility of 0.14 μg/ml. In this way, it was necessary to design or select a formulation for this drug as a means to enhance its aqueous solubility. Lipid materials were selected for preparation of the formulation as they are approved for use in humans and liposomes have proven to be effective in the delivery of anti-cancer agents.

It is established that liposomes solubilize hydrophobic drugs via incorporation of the molecules into the hydrophobic region of the bilayer [6]. Therefore, in order to achieve a stable formulation with a reasonable loading capacity for the drug of interest, it is necessary to have a high degree of miscibility between the drug and the major components of the lipid bilayer. For example, liposomal formulations for the hydrophobic anti-cancer agent paclitaxel have been explored quite extensively and in most cases PC-based lipids were employed owing to the established miscibility between paclitaxel and PC lipids [13–15, 23].

In the current study, the natural PC-based lipid, ePC, was employed as the major component with DSPE-PEG as the minor component for steric stabilization. Cholesterol was not included in the formulations as it has been shown to reduce the loading capacity and efficiency of liposomes for hydrophobic drugs [14, 18]. EPC is a mixture of saturated and unsaturated phosphatidylcholine lipids: 34% (w/w) DPPC (16:0, $T_m = 41°C$), 32% (w/w) dioleoylphosphatylcholine (18:1, $T_m = -20°C$), 18% (w/w) dilinoeoylphosphatidylcholine (18:2, $T_m = -53°C$), 11% (w/w) distearoylphosphatidylcholine (18:0, $T_m = 55°C$) and a small amount of diarachidonoylphosphatidylcholine (20:4, $T_m = -70°C$) [20]. The average transition temperature for the ePC liposomes was found to be −1.12°C as measured by DSC analysis; thus, the bilayers are fluid-like at room or physiological temperatures. The fluid nature of the bilayer should allow for the incorporation and accommodation of bulky drug molecules.

The ePC/DSPE-PEG 2000 formulation for ML220 was initially optimized in terms of the lipid concentration and the drug to lipid ratio employed for preparation. As shown in Table 1, an increase in the lipid concentration from 25 to 50 mg/ml did not significantly increase the amount of drug loaded and resulted in a decrease in the drug loading efficiency. The maximum drug concentration achieved was 3.2 mg/ml in the lipid formulation with 50 mg/ml lipid and a drug to lipid ratio of 1:7.5 (w/w). However, the drug loading efficiency for this formulation was only 48%. The preparation with a lipid concentration of 25 mg/ml and drug

to lipid ratio of 1:10 (w/w) had a loading efficiency of 85% and final drug concentration of 2 mg/ml. In addition, it was found that the concentration of drug in the formulation could be increased to 8.3 mg/ml (SD 0.5 mg/ml) following lyophilization and resuspension in HBS. Therefore, based on these results, a lipid concentration of 25 mg/ml and drug to lipid ratio of 1:10 (w/w) was employed for the formulation studies.

The composition of the formulation was also optimized by varying the ratio of ePC to DSPE-PEG 2000 (i.e., 95:5, 90:10, 80:20 mol%). As shown in Table 2, an increase in the amount of PEGylated lipid resulted in a slight decrease in both the total drug loaded and the drug loading efficiency. The addition of increasing amounts of DSPE-PEG to the formulation likely reduces the fluidity of the bilayer and, in turn, the ability to accommodate bulky drug molecules. On the other hand, the presence of increasing amounts of the PEGylated lipid allowed for preparation of a more uniform size distribution of particles. Specifically, the formulation prepared from 95% ePC to 5% DSPE-PEG included a bimodal size distribution of particles having mean diameters of 102 and 493 nm following extrusion; while, post-lyophilization the particles were 229 and 2,571 nm in diameter. The formulation prepared with 10% DSPE-PEG resulted in a unimodal distribution of particles post-extrusion but following lyophilization the particle size distribution became multi-modal. As shown in Fig. 1, when 20% DSPE-PEG 2000 was employed, the size distribution of the particles was unimodal, both prior to (diameter = 86 nm) and following the lyophilization procedure (diameter = 89 nm).

The morphology of the particles formed from ePC/DSPE-PEG 2000 80/20 (mol%) was investigated by multi-angle light scattering and TEM. As shown in Fig. 2, the average diameter of the ML220-loaded particles was approximately the same at all angles of measurement. In this way, it is demonstrated that the particles are roughly spherical in shape. Also, electron microscopic analysis revealed a spherical morphology with a relatively uniform size distribution for the liposomes (Fig. 3).

Therefore, from a consideration of the physicochemical characteristics of the drug-loaded liposomes, it was determined that a mixture of ePC/DSPE-PEG 2000 80/20 (mol%) is the optimal lipid composition for preparation of a formulation of ML220 that is suitable for i.v. administration. The formulation could be concentrated such that the level of drug loaded increased from 2 mg/ml to 8.3 mg/ml with an overall drug loading efficiency of 83%. In this way, the optimized formulation of ML220 (i.e., ePC:DSPE-PEG 80:20 mol%) resulted in more than a 50,000-fold increase in the aqueous solubility of this drug with a drug to lipid ratio of 1:3.5 (mol:mol) or 29 mol%. The extent to which this lipid formulation was able to solubilize ML220 is quite significant since most liposome formulations of hydrophobic drugs, such as paclitaxel, are only stable when the drug:lipid ratio is not increased beyond approximately 1:30 (mol:mol) [14]. The high solubilization capacity of this formulation for ML220 is likely owed to the fluid nature of the bilayer as well as a high degree of miscibility between the lipid components and the drug.

Stability of liposome formulation of ML220

The stability of the ML220 formulation was investigated in the presence and absence of physiologically relevant concentrations of BSA (45 g/l). As shown in Fig. 4, the size of the ML220-incorporated liposomes remained constant over a 3-week incubation period at 37°C in the presence and absence of BSA (i.e., 45 g/l). The stability of the liposomes is likely attributed to the adequate PEG surface coverage of the vesicles. In addition, the values obtained for the hydrodynamic diameters of the vesicles as measured in the absence and presence of BSA were compared by the student's t test and found to be insignificant ($P > 0.05$) at all time points over the 3-week period. This suggests that significant quantities of BSA do not adsorb to the surface of the liposomes.

It should be noted that self-aggregation of colloidal carriers and their interactions with serum protein have been demonstrated to be two of the key factors that influence the in vivo fate of the vehicles [24]. Following i.v. administration, the self-aggregation of colloidal carriers may cause a dramatic increase in their size and size distribution. This increase in size may, in turn, lead

Table 2 The characteristics of lipid formulations of ML220, prepared by the extrusion method, prior to and following lyophilization

Liposome formulations	Post-extrusion			Post-lyophilization		
	Diameter(nm) (mass %)	Maximum ML220 concentration (SD) (mg/ml)	Loading efficiency (SD)	Diameter(nm) (mass %)	Maximum ML220 concentration (SD) (mg/ml)	Loading efficiency (SD)
PEG2000-DSPE: ePC = 5:95	102 (96%) 493 (4%)	2.6 (0.3)	99% (2%)	229 (31%) 2571 (69%)	9.9 (0.1)	98% (1%)
PEG2000-DSPE: ePC = 10:90	86 (100%)	2.5 (0.4)	95% (5%)	104 (23%) 500 (15%) 7214 (64%)	10 (0.4)	99% (1%)
PEG2000-DSPE: ePC = 20:80	86 (100%)	2.0 (0.3)	85% (3%)	89 (100%)	8.3 (0.2)	83% (8%)

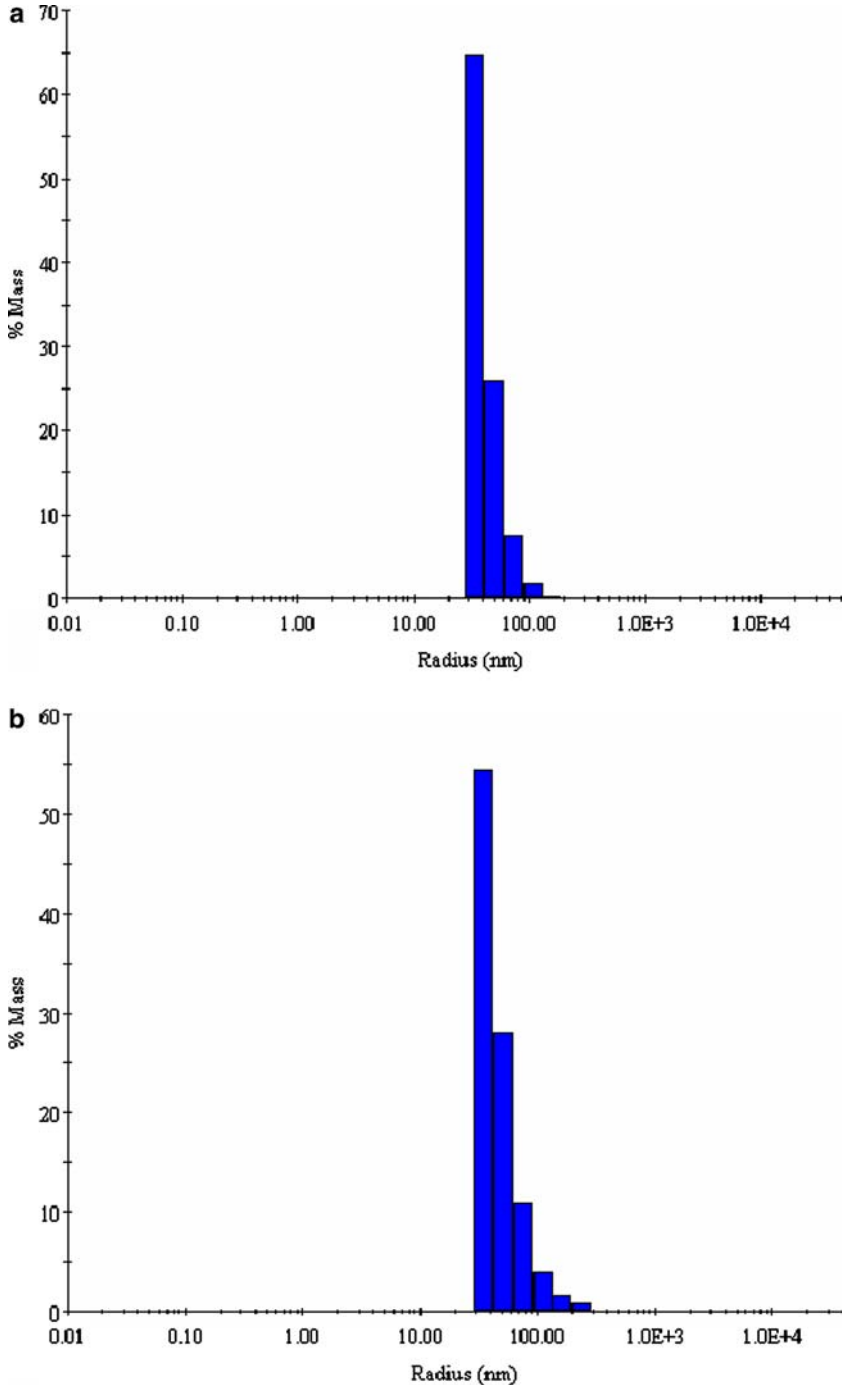

Fig. 1 Size distribution of ML220-loaded liposomes prior to (**a**) and following (**b**) lyophilization and re-suspension in HBS (0.01 M, pH = 7.4)

to a more rapid elimination of the carrier from the circulation [25, 26]. In addition, the adsorption of even trace amounts of protein has been shown to significantly influence the circulation lifetime of the vehicles [2].

Therefore, the in vitro stability of the current formulation in the absence and presence of physiologically relevant concentrations of protein suggests that this formulation may not self-aggregate or adsorb significant amounts of protein following i.v. administration. In this way, these studies provide a preliminary indication of the behavior of the formulation in vivo. However, it is realized that the in vitro stability of a delivery system or drug formulation is usually an overestimate of stability in vivo.

In vitro drug release

The release profile for ML220 from the liposomes was investigated using the dialysis method at 37°C. As shown in Fig. 5, during the first 24 h of incubation there was an initial burst release of 20% of the total drug

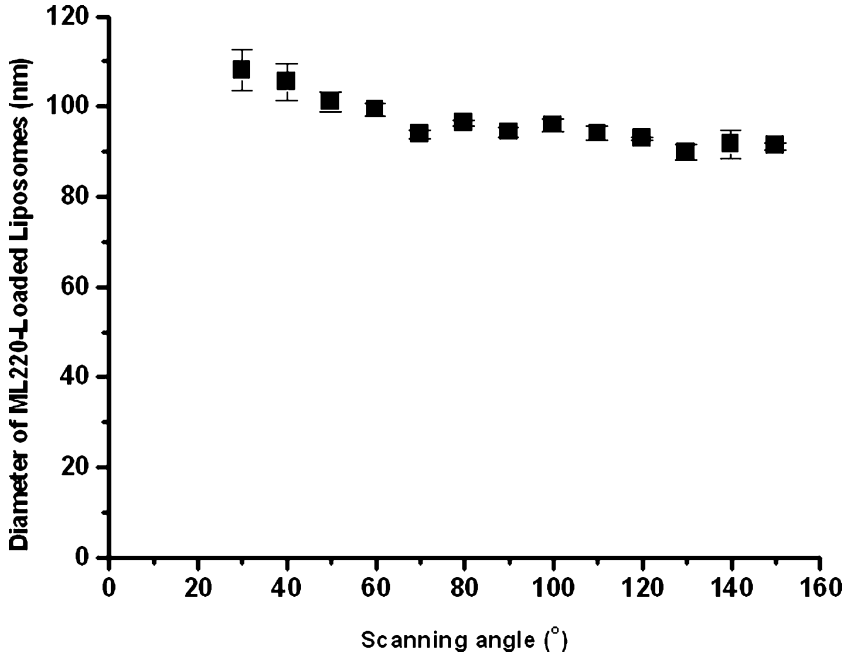

Fig. 2 Diameter of ML220-loaded liposomes as measured via multi-angle light scattering

loaded. The burst release phase was followed by a delayed release of approximately 50% of the total drug loaded over the remainder of the 2-week period.

In vitro cytotoxicity

The cytotoxic activity of ML220 formulated in liposomes was evaluated in two human cancer cell lines, SKOV-3 and MCF-7, as summarized in Tables 3 and 4, respectively. As shown, both the liposome encapsulated ML220 and free ML220 demonstrated time-dependent cytotoxicity. Also, the cytotoxicity of the liposome incorporated drug was lower than that of the free drug at all time points examined (i.e., 24, 48, 72 h) [27, 28].

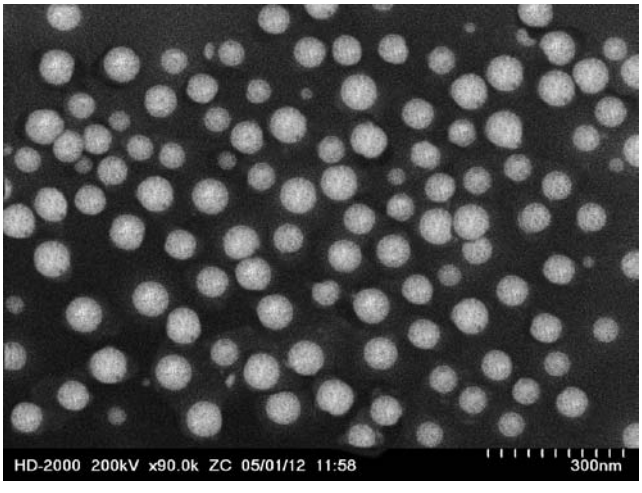

Fig. 3 Transmission electron micrograph of ML220-loaded liposomes

The reduced cytotoxicity for the liposome-incorporated drug, when compared to the free drug, is likely due to the delayed release profile for this formulation (discussed in "In vitro drug release"). In this way, the cells are not exposed to the total amount of drugs present in the liposome formulation for the entire incubation period. It should also be noted that the liposomes alone (no drug) caused no significant reduction in cell viability (data not shown).

In vivo subacute toxicity

The subacute toxicity of both the empty liposomes and the liposome formulation of ML220 were evaluated in healthy, female C3H mice. The mice were treated systemically on a daily basis with empty liposomes or a dose of the liposome encapsulated ML220 (i.e. 25, 50 and 100 mg/kg) for 3 consecutive weeks. During the 3-week period no abnormal changes in the animal's behavior (i.e., appetite, level of activity) or fur color were observed. As shown in Fig. 6, the animals in all four groups gained weight over the 3-week period. The extent of weight gain for the treatment and control groups were analyzed and compared by the student's *t* test and found to be insignificant ($P > 0.05$). Following the 3-week treatment period, all groups were sacrificed and the major organs evaluated in terms of appearance and weight. Overall, there was no significant difference between the average organ weights for the various treatment groups. These studies did not allow for determination of the maximum tolerated dose (MTD) of ML220 in the liposome formulation. Even at the highest dose given (i.e., 100 mg/kg/day ML220), no obvious adverse reactions to the treatment were observed and there was no mortality.

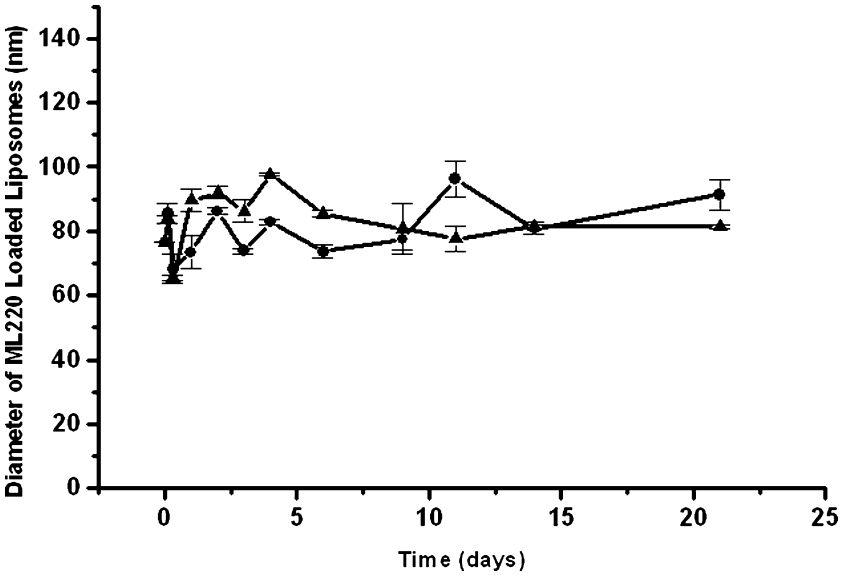

Fig. 4 In vitro stability of ML220-loaded liposomes in the absence (*filled circle*) and presence (*filled triangle*) of physiologically relevant concentrations of BSA (45 g/l)

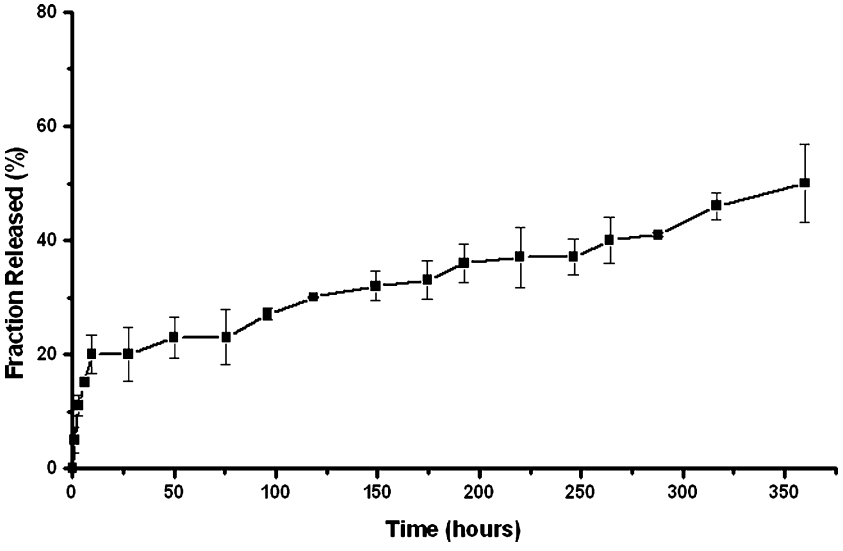

Fig. 5 In vitro release profile of ML220 from liposomes (ePC/DSPE-PEG 2000 80/20 mol%) at 37°C

Table 3 IC_{50} values (μM) for ML220 and liposome formulated ML220 in SKOV-3 cells following incubation periods of 24, 48 and 72 h

Time (h)	IC_{50} (μM)	
	Free ML220 (SD)	ML220-loaded liposome (SD)
24	9.8 (1.2)	41.7 (2.5)
48	7.4 (0.5)	26.0 (0.6)
72	5.5 (0.8)	18.4 (4.7)

Table 4 IC_{50} values (μM) for ML220 and liposome formulated ML220 in MCF-7 cells following incubation periods of 24, 48 and 72 h

Time (h)	IC_{50} (μM)	
	Free ML220 (SD)	ML220-loaded liposome (SD)
24	13.6 (1.2)	110.0 (5.4)
48	9.5 (1.1)	47.2 (4.8)
72	7.3 (0.9)	39.0 (4.1)

In vivo pharmacokinetics of ML220 formulated in liposomes

In this study, the blood clearance profile for both the liposomal lipid and the drug ML220 were investigated up to 24 h following i.v. administration. Considering the plasma volume in mice (45.6 ml/kg [29]), the volume of distribution for the liposomes (1.14 ml) was found to be very close to the total plasma volume. This indicates that the size of the liposomes allows the vehicle to be effectively trapped within the blood stream. As shown in Fig. 7a, the plasma clearance profile for the liposomal lipid demonstrates a prolonged circulation lifetime for the carrier in vivo. Specifically, more than 50% of the total lipid injected was retained within plasma up to 12 h

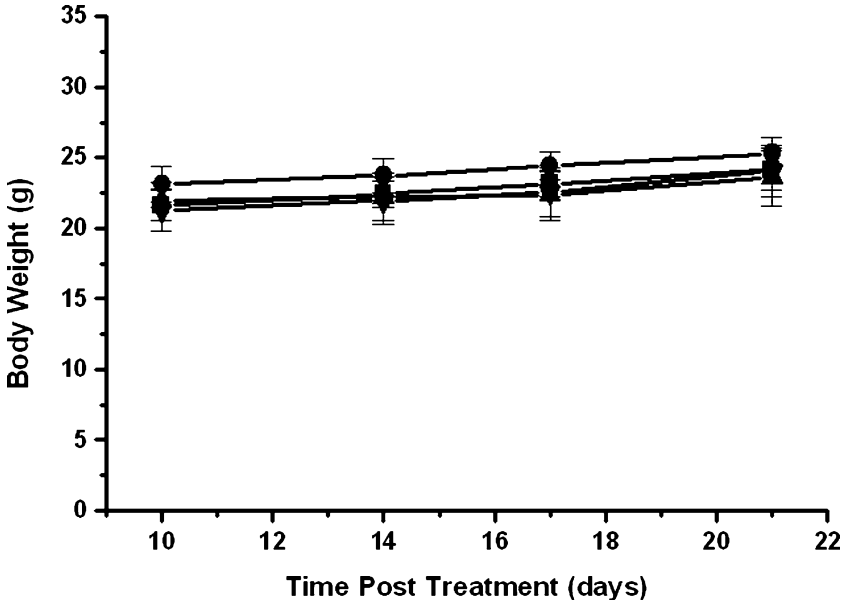

Fig. 6 Mean body weight of C3H mice following treatment with different doses of ML220 administered in liposomes (ePC/DSPE-PEG 2000). (*filled square* control, *filled circle* 25 mg ML220/kg, *filled triangle* 50 mg ML220/kg, *filled inverted triangle* 100 mg ML220/kg)

following administration, and 36% remained in the circulation following 24 h. In this way, the circulation lifetime for this carrier is equivalent to or greater than that of other nano-sized colloidal systems that have been examined to date for delivery of hydrophobic drugs [30–32]. The relatively long circulation lifetime is in agreement with the high degree of stability observed for liposomes in vitro in both the absence and presence of BSA (as discussed in "Stability of liposome formulation of ML220").

By contrast, the blood clearance profile for ML220 follows an obvious biexponential pattern with an initial fast elimination of drug in the first half hour followed by a relatively slower phase of elimination as shown in Fig. 7b. The i.v. administration of the liposome formulation of ML220 to Balb/C at a dose of 75 mg/kg resulted in an $AUC_{0-\infty}$ of 1,447 mg h/l and a $t_{1/2\ \beta}$ of 4.1 h. The mean residence time (MRT) for ML220 was determined to be 5.0 h while the total clearance CL_t was 1 ml/h. The steady state volume (V_{ss}) of ML220 was found to be 5.3 ml (i.e., 0.266 l/kg) which suggests a broad tissue distribution for this drug following i.v. administration. This broad distribution of the drug may be one of the major factors that leads to the initial fast elimination of ML220 from the plasma. Calculation of the ratio of ML220 to liposomal lipid present in the plasma allowed for the in vivo release kinetics profile for the drug from the liposomes to be determined. As shown in Fig. 7c, the in vivo release profile for ML220 from the liposomes includes an initial burst release phase in the first half hour followed by a delayed release phase over the remainder of the 24-h period. Therefore, the in vitro and in vivo drug release profiles are in agreement in terms of the length of the burst release phase relative to the length of delayed release phase.

This pharmacokinetics study highlights the two critical parameters that influence the biological performance of liposomes as drug delivery systems [33]: (1) the circulation lifetime of the carrier (2) and the ability of the carrier to retain the drug [33]. For the present liposome formulation, although the liposomes remain stable in the presence of physiologically relevant concentrations of protein, their ability to retain the drug is limited. The relatively rapid release of the drug from the liposomes in vivo may be attributed to the high protein binding affinity of this compound. The aqueous solubility of ML220 in the presence of physiologically relevant concentrations of albumin (i.e., 45 g/l) was found to be 153 μg/ml; while, the aqueous solubility in the absence of protein was 0.14 μg/ml. The more than 1,000-fold increase in the aqueous solubility of ML220 in the presence of protein demonstrates the high protein binding affinity of this drug.

It should be noted that in previous studies, it has been demonstrated that formulations resulting in a similar pharmacokinetic profile for a hydrophobic drug provide a significant improvement in the accumulation of drug at the tumor site [34]. Therefore, despite the initial fast elimination phase for this drug from the circulation, this liposomal formulation is still expected to improve the biological performance of this drug in vivo.

In vivo tissue distribution

The tissue distribution of ML220 at specific time points following administration is shown in Fig. 8. ML220 was found to accumulate to the greatest extent in the liver with a peak level observed at 0.5 h (21.4% of dose; SD = 2.3%) following injection. The degree of liver accumulation then falls off, gradually reaching 0.4% at 24 h. A significant fraction of ML220 was also found to accumulate rapidly in the heart with the maximum level achieved within 1 h (13.1% of dose; SD = 0.9%) fol-

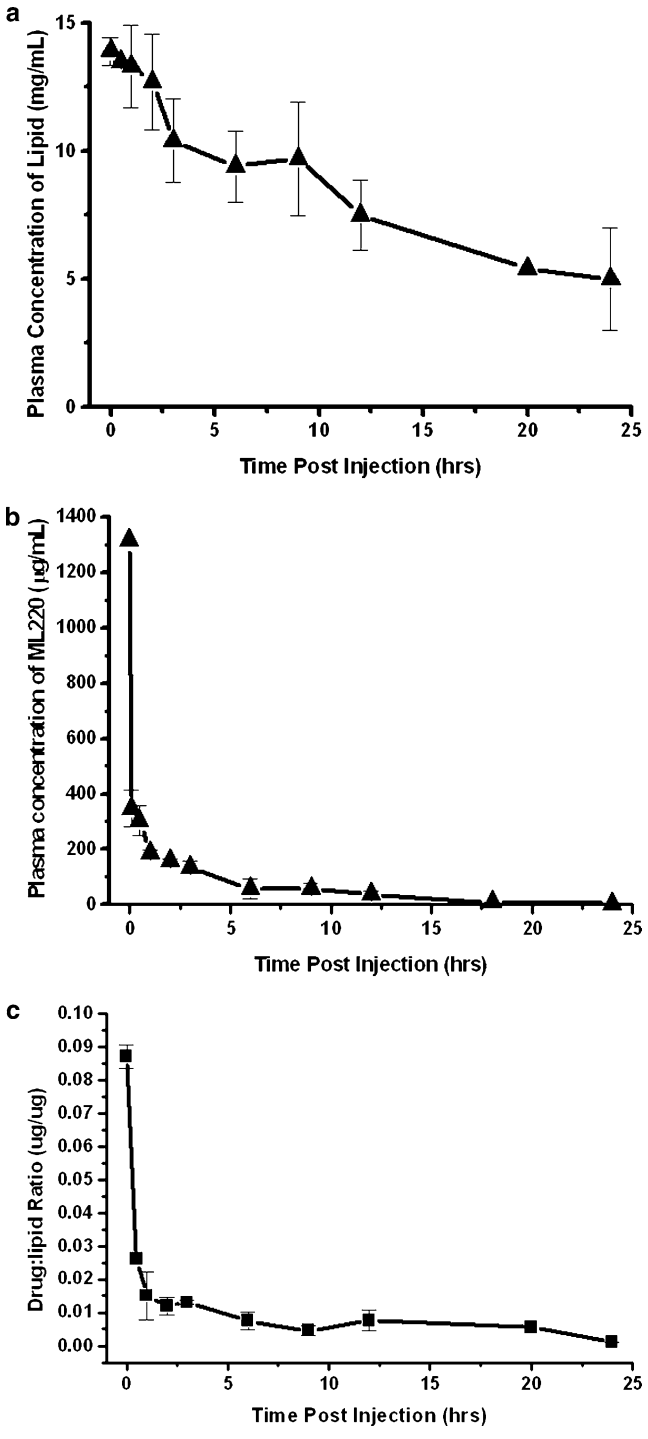

Fig. 7 Plasma concentration of liposomal lipid (**a**), ML220 (**b**) and ratio of ML220 to lipid (**c**) following i.v. administration of the ePC/DSPE-PEG 2000 liposome formulation of ML220 (i.e. 75 mg/kg ML220) in Balb/C mice ($n = 3$). At the indicated times, the plasma concentration of ML220 was measured by HPLC and lipid concentration was determined by liquid scintillation counting

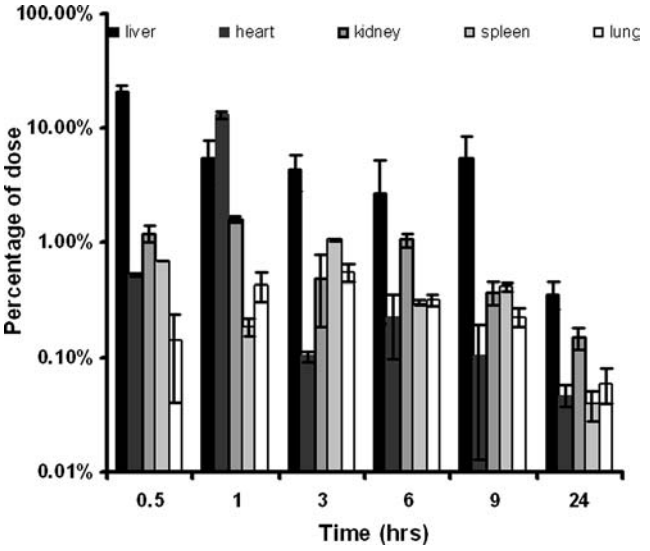

Fig. 8 Tissue distribution of ML220 in mice following i.v. administration of the ePC/DSPE-PEG 2000 liposome formulated ML220. The amount of ML220 is expressed as the percentage of the injected dose versus time. The histograms are representative of the mean of three independent determinations ($n = 3$)

lowing injection. The accumulation in the heart was found to decline rapidly following the 1-h time point. The lowest level of accumulation for this drug was found in the lung, which was characterized by both a slow rate of accumulation and elimination. The AUC values in all organs examined from 0 to 24 h decreased in the following order: liver > heart > kidney > spleen > lung. The results obtained for the tissue distribution of the drug correlate well with the blood clearance profile for this agent. A significant amount of drug distributes quickly to the liver and heart and thus induces rapid elimination of the drug from the plasma as discussed in "In vivo pharmacokinetics of ML220 formulated in liposomes".

In vivo anti-tumor efficacy

The anti-tumor activity of ML220 formulated in liposomes was investigated in a human colon HT-29 carcinoma model in nude CD-1 mice. Approximately 5 days following subcutaneous implantation of the HT-29 colon cancer cells, the tumors were found to become well-established (i.e., mean volume between 50 and 200 mm^3). Figure 9 includes a plot of the relative tumor volume post-treatment as a function of time. The tumor growth inhibition was evaluated by comparing the relative tumor volume between the treatment group (i.e., 75 mg/kg/day ML220 formulated in liposomes) and the control group (i.e., HBS treatment). The difference between the two groups was analyzed by the student's *t* test and found to be significant ($P < 0.05$) at all time points with the exception of the second day post-treatment (i.e., 6, 9, 13 and 16 days). Therefore, it has been demonstrated that ML220 administered at a dose of 75 mg/kg/day in the lipid formulation provides a significant delay

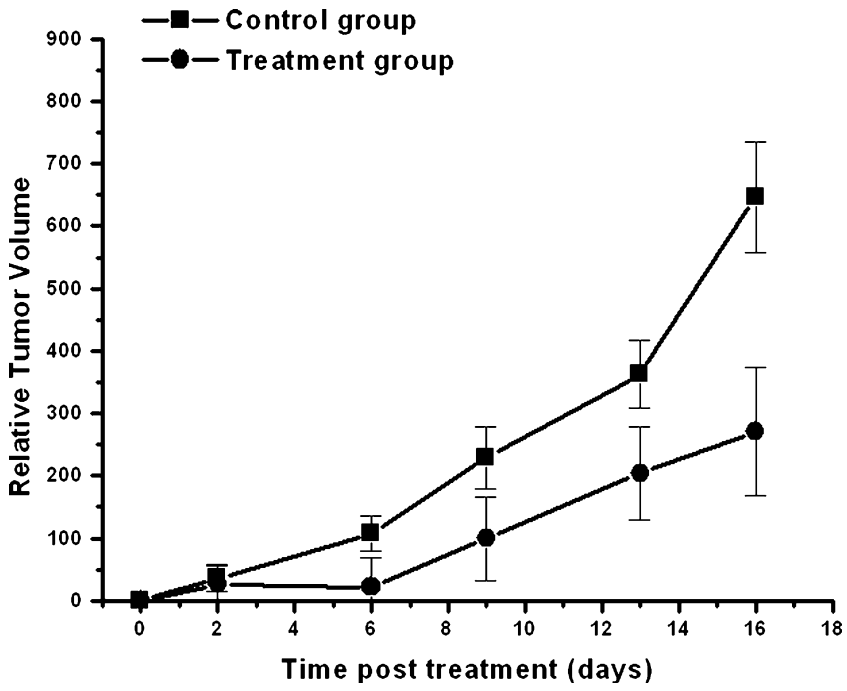

Fig. 9 In vivo anti-tumor activity of ML220 administered in liposomes in a human HT-29 colon tumor xenograft model in CD-1 nude mice. Control group (saline; $n = 5$) and treatment group (100 mg ML220/kg/day, $n = 5$)

in tumor growth, compared with that for saline, in the HT-29 human colon carcinoma model. It should also be noted that there was no mortality in the treatment group and the MTD of this formulation (i.e., higher than 100 mg/kg/day ML220) has been demonstrated to be much higher than the dose employed in this efficacy study. Due to the relatively low toxicity of the formulated ML220, higher doses could be employed in future studies and may lead to more significant delays in tumor growth.

Conclusion

The design of a delivery system for ML220 is particularly challenging owing to its limited water solubility (i.e., 0.14 μg/ml). These studies demonstrate that the fluid-like, cholesterol-free ePC-based liposomes have an especially high solubilization capacity for this drug. These preliminary findings are promising and support further evaluation of this formulation for ML220 and its hydrophobic analogues.

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