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Liposomal topotecan formulation with a low polyethylene glycol grafting density: pharmacokinetics and antitumour activity

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Abstract

Objectives PEGylated liposomes could evade recognition by the reticulo-endothelial system and prolong the circulation time of vesicles, resulting in enhanced targeting efficiency and antitumour effect. Typically, vesicles are modified with distearoylphosphatidylethanolamine (DSPE)-polyethylene glycol (PEG) at a high PEG grafting density. However, long circulation time and slow drug release rate might induce severe hand-foot syndrome in clinical practice. In this study, a liposomal topotecan formulation with a low PEG grafting density was prepared and its pharmacokinetics, acute toxicity and antitumour effect were investigated.

Methods Topotecan was loaded into liposomes using an ammonium sulfate gradient. The resulting formulation was injected to healthy Wistar rats at different dose levels to investigate whether its clearance followed linear kinetics. Biodistribution was performed in Lewis lung cancer-bearing mice. The acute toxicity was evaluated in healthy mice and beagle dogs. To compare the antitumour effects of different formulations and dose schedule, RM-1 prostate, Lewis lung, H446 and L1210 cancer models were used.

Key findings Topotecan could be encapsulated into low DSPE-PEG liposomes with ~100% loading efficiency. The clearance of the liposomal formulation followed linear kinetics at a dose level ranging from 0.5 to 4 mg/kg despite the fact that the vesicles were coated at a low PEG density. Compared with free topotecan the liposomal formulation preferentially accumulated into tumour zones instead of normal tissues. Both formulations could rapidly accumulate into liver and tumour, but the liposomal formulation was cleared from tissues at a slow rate relative to the conventional formulation. In rats and beagle dogs, liposomal formulations could not induce skin toxicity. In all the tumour models, smaller split doses were more therapeutically active than larger doses when the overall dose intensity was equivalent.

Conclusions This has been the first report that plasma kinetics of a liposomal formulation with a low PEG density followed linear kinetics. Moreover, due to its short circulation half-life, the formulation did not induce skin toxicity. Our data revealed that the dose schedule of liposomal drugs should be adjusted in accordance with the biophysical and biological properties of the formulations to achieve the optimal therapeutic efficacy.

Introduction

The use of liposomes as drug carriers for chemotherapeutic agents offers a potential means of manipulating drug pharmacokinetics and biodistribution to improve antitumour efficacy and reduce toxicity.[1] The degree to which the pharmacokinetics and biodistribution of the drug are affected relies on three variables: the composition of the lipid bilayer and liposomal water compartment, namely, liposome types; the properties of the drug; and the nature of the interaction between the drug and the lipid vesicle compartments.[1–4] In the past 30 years, various types of liposomes have been

designed to optimize the delivery of antitumour drugs. The breakthroughs in the liposome field have led to the approval of four liposomal antitumour drug formulations including Myocet, DaunoXome, Lipoplatin and Doxil.[4–7] The former two are conventional liposomes and the latter two are PEGylated liposomal drugs.

Conventional liposomes are made from solid (or fluid) phosphatidylcholines and cholesterol.[1] Following intravenous injection, they are easily recognized and rapidly cleared from circulation by the reticulo-endothelial system (RES).[1] To resolve this problem, PEGylated liposomes were introduced in the late 1980s.[1,8] The surfaces of the PEGylated liposomes are coated with polyethylene glycol (PEG), a synthetic hydrophilic polymer, and thus they could effectively evade the recognition and clearance by the RES, resulting in long circulation time. To what degree the circulation time of the vesicles is prolonged relies on PEG grafting density and molecular weight of PEG. Typically, negatively-charged polymer distearoylphosphatidylethanolamine (DSPE)-PEG with a mean PEG molecular weight of 2000 is used at a high PEG grafting density (~8 mol%, DSPE-PEG/phosphatidylcholines (PCs)).[1,8]

Despite the fact that high PEG grafting density could significantly prolong circulation time, it might induce undesirable clinical side effects such as hand-foot syndrome, especially when the formulation has a slow drug leakage rate (e.g. Doxil, a PEGylated liposomal doxorubicin formulation loaded with ammonium sulfate gradient).[4,5,9] The mechanism underlying why hand-foot syndrome could occur is proposed as follows: if the PEGylated liposomes could circulate long enough in blood, they could accumulate into the terminal of limbs and skin, where if they could effectively extravasate capillary blood vessels and release entrapped cytotoxic drugs, hand-foot syndrome might occur. Accordingly, long circulation time and stable drug encapsulation are two necessary conditions for the occurrence of hand-foot syndrome. The toxicity should be attributed to the combination of the carrier and the drug. How to lessen or eliminate the side effect? One answer is to use a formulation with a low PEG grafting density to reduce vesicle circulation time and the other is to develop the liposomal formulation with a fast drug release rate.

The decrease of PEG grafting density and increasing drug release rate might affect the pharmacokinetics and biodistribution of liposomal drugs. Thus, a series of questions arise. Whether the clearance of such formulations follows linear plasma kinetics? Does the decrease of PEG grafting density affect the half-life of liposomal drugs and then affect the selection of dose schedule? Could the formulation induce hand-foot syndrome?

Topotecan is a water-soluble camptothecin derivative, which has been indicated for the treatment of solid tumours (e.g. metastatic carcinoma of the ovary, small cell lung cancer and stage IV-B, recurrent, or persistent carcinoma of the cervix) and leukaemia.[10–13] Like other camptothecins, topotecan is one of the topoisomerase I inhibitors, which could bind to topoisomerase I-DNA complex, resulting in double-strand DNA damage and cell death. Due to its toxicity and insolubility camptothecin is not used in clinical practice now and thus a series of water soluble derivatives of camptothecin have been synthesized, of which only two drugs have been approved (topotecan and irinotecan). Topotecan is more therapeutically active than irinotecan. Liposomal delivery of topotecan could provide protection against drug hydrolysis, deliver more active lactone form to the tumour and prolong topotecan exposure time.[14–16]

In this study, a PEGylated liposomal topotecan formulation with a low PEG density (3.0 mol% relative to PCs) was prepared using the ammonium sulfate gradient method. The plasma pharmacokinetics of the formulation was investigated in normal rats at different dose levels to determine whether it followed linear clearance kinetics. To investigate the effect of the dose schedule on therapeutic activity of the liposome formulations, Lewis lung cancer, RM-1 prostate cancer, H446 human lung carcinoma and L1210 liver metastasis models were used. The skin toxicity of the formulation was evaluated using beagle dogs as the animal model. This study has been the first attempt to investigate the pharmacokinetics, skin toxicity and optimum dose schedule of liposomal formulations with a low PEG grafting density. Meanwhile, a standard high PEG density formulation was prepared and evaluated as a comparison.

Materials and Methods

Materials

Topotecan hydrochloride was provided by Chengdu Tianyuan Natural Product Co., Ltd (Chengdu, China). Hydrogenated soybean phosphatidylcholine (HSPC) was a kind gift from Degussa (Freising, Germany). N-(Carbonyl-methoxypolyethyleneglycol$_{2000}$)-1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine, sodium salt (MPEG$_{2000}$-DSPE) was obtained from Genzyme Pharmaceuticals (Liestal, Switzerland). Cholesterol and Sephadex G-75 (medium) were obtained from the Sigma Chemical Company (St Louis, MO, USA). Nucleopore polycarbonate filters (47 mm, 0.1 μm pore sizes) were obtained from Northernlipids, Inc. (Burnaby, BC, Canada). All other chemicals used in this study were analytical or high-performance liquid chromatography (HPLC) grade.

The RM-1 (mouse prostate tumour), L1210 (mouse leukaemia), H446 (human lung carcinoma) and Lewis lung cancer cell lines were originally purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Wistar rats and beagle dogs were obtained from Hebei Medical University. Kunming, BDF1, C57 and Nu/Nu nude mice were purchased from Vitalriver (Beijing, China).

Preparation of liposomes

Liposomes were prepared according to the following procedure. Briefly, the mixtures of HSPC, cholesterol and $MPEG_{2000}$-DSPE (59.26 : 38.96 : 1.78, molar ratio; PEG grafting density, 3.0%) were solubilized in chloroform and dried to a thin lipid film under a stream of N_2 gas, followed by incubation overnight under vacuum to remove residual solvent. The dried lipid films were subsequently hydrated with 250 mM ammonium sulfate. The hydration process was performed at 60°C for 1 h. The dispersion was extruded eight times through 0.10-μm polycarbonate filters employing a LiposoFast-100 jacketed extruder obtained from Avestin (Ottawa, Canada) at 60°C. This procedure formed unilamellar vesicles of ~100 nm.

The average size of vesicles was analysed using quasi-elastic light scattering (Zetasizer Nano ZS; Malvern Instruments, Malvern, UK). Before analysis the samples were diluted in 0.9% NaCl with a volume ratio of 1/200. The zeta potential of vesicles was determined using Nano ZS, but the measurement was carried out in water after 50-fold dilution. DTS 4.0 software was used to collect the data that were analysed using 'multi-narrow modes'.

The standard high PEG density formulation was prepared and characterized using the same procedure, in which the molar ratio of HSPC to cholesterol was kept unchanged (3:2), but the PEG grafting density was elevated (8.0 molar% relative to HSPC).

Remote loading of liposomes

A transmembrane ion gradient was generated across the vesicles by exchanging the extraliposomal buffer using Sephadex G-75 columns. The buffer employed in the experiments was pH 6.0 sucrose/histidine (300 / 20, mM/mM) buffer. Upon buffer exchange, empty liposomes with a transmembrane ion gradient were mixed with topotecan at a mass ratio of 28 : 1. The resulting mixture was incubated at 60°C for 40 min to realize drug loading.

For determination of the loading efficiency, samples of the mixtures were taken and unentrapped topotecan was removed by size exclusion chromatography. Briefly, 100-μl samples were loaded onto a Sephadex G-75 mini-column (56 × 8 mm i.d.), and then eluted using 0.9% NaCl solution. The loading was calculated using the formula: loading % = liposome fraction/(liposome fraction + free drug fraction) × 100.

Animal experiments

All animal experiment protocols were approved by CSPC ZhongQi Institutional Animal Care and Use Committee (approval number: 2010-PLT-01, 2010-PLT-02, 2010-PLT-03, 2011-PLT-01; approval date: December 15, 2010 and September 1, 2011) and complied with Regulations for the Administration of Affairs concerning Experimental Animals (Hebei Province, China).

Pharmacokinetic and biodistribution studies

Plasma pharmacokinetic analysis was performed in normal Wistar rats. Thirty Wistar rats were randomly divided into five treatment groups with six rats in each group (male : female, 1 : 1). Each group received a single intravenous bolus dose of liposomal or free topotecan via the tail vein (liposomal topotecan with a low PEG density: 0.5, 1, 2 or 4 mg/kg; topotecan solution: 4 mg/kg). The topotecan solution (0.4 mg topotecan/ml) was prepared by dissolving topotecan hydrochloride in 5% glucose and the pH was adjusted to ~4 to prevent the formation of the inactive carboxylate form. At specified time points blood samples were obtained and collected in Eppendorf tubes containing sodium heparin as an anticoagulant. Blood samples were centrifuged at 600*g* for 10 min to separate the plasma. The plasma samples were stored at −20°C until additional analysis.

To compare the pharmacokinetics of the low PEG formulation with that of the standard formulation, both formulations were injected into male Wistar rats at a dose of 2 mg/kg (three rats per group × two groups) and the plasma samples were collected using the same procedure.

Biodistribution studies were performed in healthy male C57 mice bearing Lewis lung cancer (tumour weight: 0.25–0.30 g). Forty eight mice were divided into two groups: one group received 5 mg/kg liposomal (low PEG formulation) topotecan and the second group received free topotecan. At the indicated time points (1, 4, 8 and 24 h), mice were killed by cervical dislocation (six mice per time point per group) and tissues were rapidly excised, rinsed in ice-cooled normal saline and snap frozen.

Topotecan concentrations in plasma were determined using a well-established HPLC method.[16] Before analysis, mouse tissues were homogenized using a Tissue-Tearor equipped with a 7 mm probe (Biospec Products, Inc., Bartlesville, OK, USA). A 20% (w/v) homogenate was prepared in cold purified water.

For 50 μl plasma, 950 μl methanol containing 0.2% v/v acetic acid (–20°C) was added. For 100 μl homogenate, 500 μl 0.2% v/v acetic acid/methanol solution (–20°C) was added. The resulting mixture was vortexed for 30 s and then centrifuged at 20 000*g* for 10 min (–10°C). The supernatant was collected for analysis. The injection volume for samples was 20 μl.

A Shimadzu HPLC system controlled by LC solution software was used for chromatographic analysis, which was composed of DGU-20A5 degasser, LC-20AT liquid chromatograph, SIL-20A autosampler, RF-10AXL detector and CTO-20A column oven. The autosampler and the column compartment were maintained at 25°C. The HPLC separations were achieved using a Diamonsil C18 column

(150 × 4 mm i.d., 5 μm particle size) from Dikma with a run time of 15 min at a flow rate of 1 ml/min. A guard column (Diamonisl C18, 4 × 8 mm) was installed ahead of the analytical column. The ex/em wave lengths were 381/525 nm, respectively.

The mobile phase was a mixture of acetonitrile (A) and aqueous phase (B) (3% triethylammonium solution adjusted to pH 5.5 with acetic acid (23 : 77, v/v)). Under the HPLC conditions outlined above, topotecan were eluted at 7 min. The recovery of topotecan was > 95% and the standard curve with an *r*-value of 0.999.

Antitumour efficacy study

To evaluate the antitumour effect of different formulations and dose schedules, three tumour models were employed.

RM-1, H446 and Lewis lung cancer models

To set up RM-1 prostate tumour, tumour cells were injected subcutaneously (2.5×10^6 cell per mouse) in the right flank region of female BDF1 mice. The Lewis lung cancer (LLC) model was established by the subcutaneous injection of 5×10^5 cell per mouse in the right flank region of male C57 mice. In both cases, tumours were allowed to grow to a mean tumour volume of ~0.3 cm^3 before the initiation of treatment. Tumour-bearing mice were randomly divided into four groups ($n = 11$ for RM-1; $n = 10$ for LLC model). Liposomal topotecan (low PEG formulation) was administrated to mice as a single large dose (8 mg/kg) or as two small split doses (4 mg/kg every seven days for RM-1 tumour; 4 mg/kg every four days for LLC model). Free topotecan was administrated as a single dose at a level of 8 mg/kg. Control mice were treated with an isotonic sucrose-histidine solution.

In contrast, H446 human lung carcinoma model was established by the subcutaneous injection of ~6×10^6 cell per mouse in the right flank region of male Nu/Nu nude mice. The treatment was initialized until the mean tumour volume reached ~110 mm^3. The mice were randomly divided into eight groups (nine mice per group). The groups received low PEG liposomal topotecan (2 mg/kg/quaque 1 (q1) week × 4, 1 mg/kg/q1 week × 4, 0.5 mg/kg/q1 week × 4, 4 mg/kg/quaque 2 (q2) weeks × 2 or 1 mg/kg/quaque 0.5 (q0.5) week × 8), free topotecan (2 mg/kg/q1 weeks × 4 or 4 mg/kg/q2 weeks × 2) or control solution (an isotonic sucrose-histidine solution), respectively.

In all cases, the tumour size was measured using vernier calipers. Tumour volume (V) was calculated according to the equation $(\pi/6) \times \text{width}^2 \times \text{length}$. Animal weight and tumour size were monitored by qualified technicians.

L1210 liver metastasis model

Male BDF1 mice were inoculated intravenously with 5×10^4 L1210 murine tumour cells, derived from the ascitic fluid of a previously infected BDF1 mouse. Free topotecan or liposome-encapsulated topotecan (low PEG formulation) were administrated via a lateral tail vein, 24 h after tumour cell inoculation. Animal weights were monitored daily and mortality was determined up to 60 days. Death cannot be used as an end point and so mice were killed at the first sign of distress. The data were analysed with SPSS 11.5 version software (survival analysis).

Acute toxicity evaluation

The maximum tolerated dose of liposomal topotecan formulations (both high and low PEG density formulations) following intravenous administration was evaluated in healthy Kunming mice. Briefly, the drug was administrated via the tail vein in groups of two mice, beginning with 10 mg/kg topotecan and continuing with a dose escalation factor of ~1.2 until a dose level of 25 mg/kg was achieved (dose levels: 10, 12, 14.4, 17.3, 20.7 and 24.9 mg/kg). If during the observation period there was no mortality, irreversible morbidity, or severe body weight loss (consistent loss in excess of 20% of original weight maintained for 72 h), the highest administered dose was considered as the acute single injection maximum tolerated dose.

In all cases, qualified animal care technicians monitored the mice for weight loss and other signs of stress/toxicity (e.g. decrease in food uptake and activity) for a period of 21 days. Death cannot be used as an end point and so mice were killed by cervical dislocation at the first sign of distress for humane consideration. After 21 days, all remaining animals were killed by cervical dislocation and necropsies were conducted to identify any additional drug toxicities.

To evaluate the skin toxicity of liposomal topotecan, the low PEG formulation was administrated to beagle dogs at three dose levels (3.2, 6.4 and 12.8 mg/m^2) and the standard PEG formulation was injected at a dose level of 6.4 mg/m^2. One group contained two dogs (one female and one male). Weight loss and other signs of toxicity were monitored for four weeks and then the animals were killed by intravenous injection of pentobarbital to identify any drug toxicity.

Drug release studies

Topotecan release from different vesicles was monitored using a fluorescence dequenching assay. Before analysis, a sample of liposomal topotecan was diluted 1000-fold by injection into a quartz cuvette containing a solution of glucose 250 mM, histidine 10 mM and NH_4Cl 20 mM, pH 7.5. A Hitachi F-4600 fluorescence spectrophotometer was employed. The fluorescence intensity data were collected continually with a time interval of ~8 s. The ex/em wavelengths were 381/525 nm, respectively. Temperature was controlled at 37°C using a jacketed sample holder, which was connected to a circulating water bath. The achieved intensity

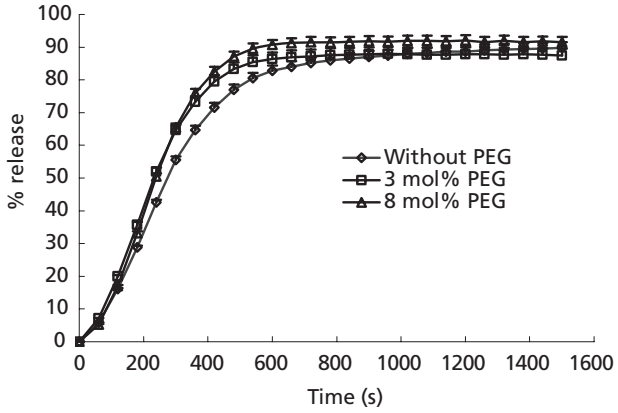

Figure 1 Effect of polyethylene glycol grafting density on drug release rate. Data points represent the mean values calculated from six repeated measures. For comparison the fluorescence intensity was converted to % release. PEG, polyethylene glycol.

data were converted to concentration data according to a fluorescence intensity (FI) vs concentration standard curve. % release was determined by $100 \times ([topo]_t - [topo]_0)/[topo]_{total}$, where $[topo]_t$ and $[topo]_0$ are free topotecan concentrations at time points t and 0, $[topo]_{total}$ is the total topotecan concentration, including both free and liposomal topotecan. To quantitatively compare the difference in drug release kinetics, % release was plotted as a function of time.

Statistical analysis

The results in all figures and tables are shown as the mean ± standard deviation (SD). The data at each sampling point in Figures 1, 2 and 4–6 were statistically examined using the Kruskal-Wallis test. Individual differences between the various formulations were then examined using Nemenyi's test. The data in Figure 3 was analysed using the Mann-Whitney U test. In all cases, $P < 0.05$ was considered to be statistically significant.

Results

The characterization of liposomal topotecan formulation

Topotecan was loaded into PEGylated liposomes exhibiting a transmembrane ammonium sulfate gradient.[17] Before drug loading, empty vesicles with entrapped ammonium sulfate were exchanged into a pH 6.0 sucrose/histidine buffer. The drug loading efficiency was ~100% and the resulting drug/lipid mass ratio was 1 : 28 for both formulations (low PEG and standard PEG formulation). After drug loading, the vesicles still had a narrow size distribution (98.7 ± 1.4 and 99.6 ± 2.1 nm for low and high PEG formulations,

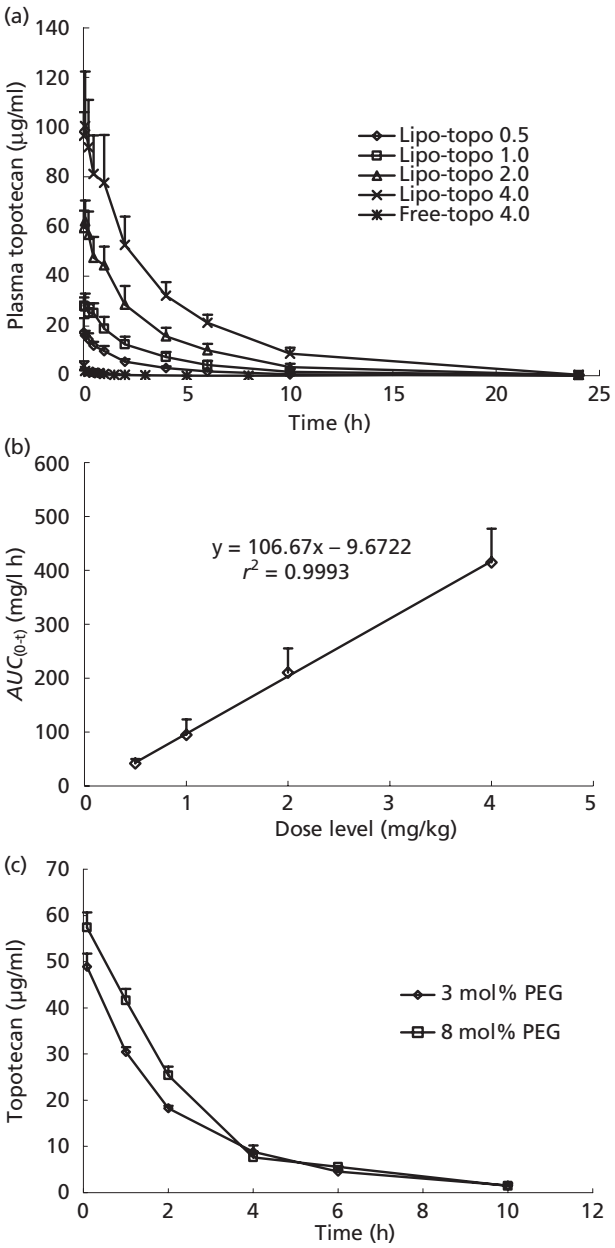

Figure 2 Plasma pharmacokinetics of liposomal and free topotecan in Wistar rats. (a) Liposomal topotecan (Lipo-topo) with a low polyethylene glycol (PEG) content was intravenously administrated at 0.5, 1, 2 and 4 mg/kg and free topotecan was injected at 4 mg/kg (Free-topo; $n = 6$). (b) Dose dependence of liposomal topotecan (low PEG formulation). The plasma area under the curve (*AUC*) following intravenous administration of liposomal topotecan was plotted vs dose level. (c) The comparison of pharmacokinetic profile of a low PEG formulation (3 mol% PEG) with that of a standard PEG formulation (8 mol% PEG) at a dose level of 2 mg/kg.

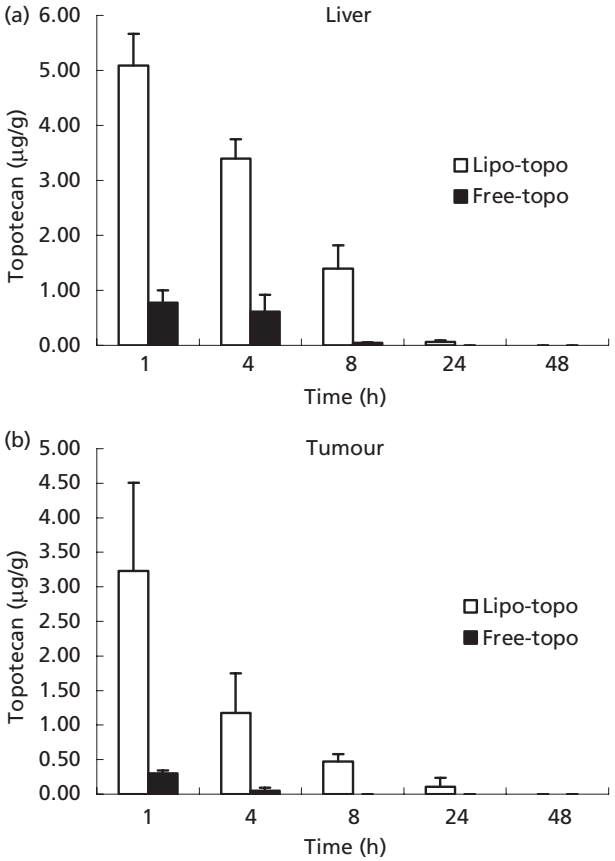

Figure 3 Topotecan levels in liver and tumor following the injection of liposomal (low polyethylene glycol formulation) and free topotecan formulations in Lewis lung cancer bearing C57 mice. Liposomal topotecan, Lipo-top; free topotecan, Free-topo. Both formulations were injected at a dose level of 5 mg/kg ($n = 6$).

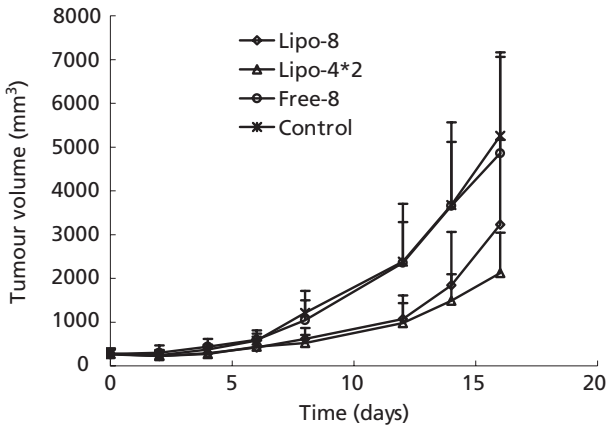

Figure 4 Antitumour effect of liposomal (low polyethylene glycol formulation) and free topotecan in RM-1 prostate tumour bearing female BDF1 mice. When the mean tumour volume reached to ~0.27 cm^3, topotecan formulations were injected into mice at different dose schedules ($n = 11$). Liposomal topotecan: a single injection at 8 mg/kg (Lipo-8), two doses of 4 mg/kg administrated every six days (Lipo-4*2); free topotecan: a single dose of 8 mg/kg (Free-8).

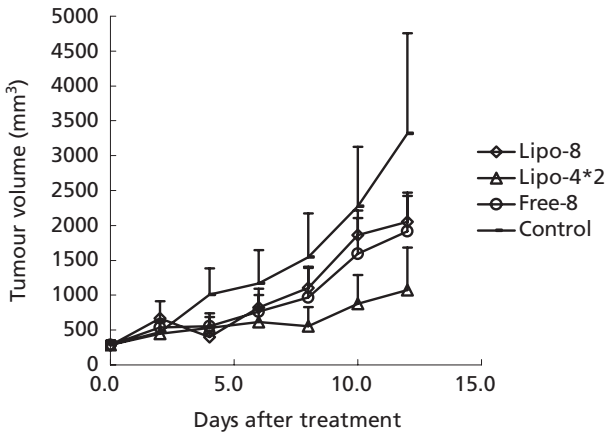

Figure 5 Antitumour effect of liposomal (low polyethylene glycol formulation) and free topotecan in Lewis lung cancer bearing male C57 mice. When the mean tumour volume reached to ~0.29 cm^3, topotecan formulations were injected into mice at different dose schedules ($n = 10$). Liposomal topotecan: a single injection at 8 mg/kg (Lipo-8), two doses of 4 mg/kg administrated every six days (Lipo-4*2); free topotecan: a single dose of 8 mg/kg (Free-8).

respectively), with a polydispersion index of ~0.05. Zeta potential measurement showed that both formulations were negatively charged with a zeta potential of -28.7 ± 1.9 and -35.6 ± 1.7 mV, respectively. Since the PEGylated lipid used was negatively charged at physiological pH, the high DSPE-PEG formulation carried more charges than the formulation with a low PEG content.

Drug release

To investigate drug release profiles of different formulations, drug release experiments were performed in NH_3-containing release media. Free NH_3 could freely permeate the lipid bilayer and elevate intraliposomal pH, thus inducing drug release. Based on data presented in Figure 1, the drug release rates for both PEGylated formulations were similar and the conventional non-PEGylated formulation had a slow drug release rate compared with the PEGylated formulations. It seemed that the modification of vesicles with PEGylated lipids could slightly increase the drug leakage, which might have been associated with the electrostatic interaction of positively-charged topotecan with negatively-charged DSPE-PEG.

Acute toxicity

Acute toxicity studies were performed in healthy Kunming mice and beagle dogs. Liposomal topotecan with a low PEG

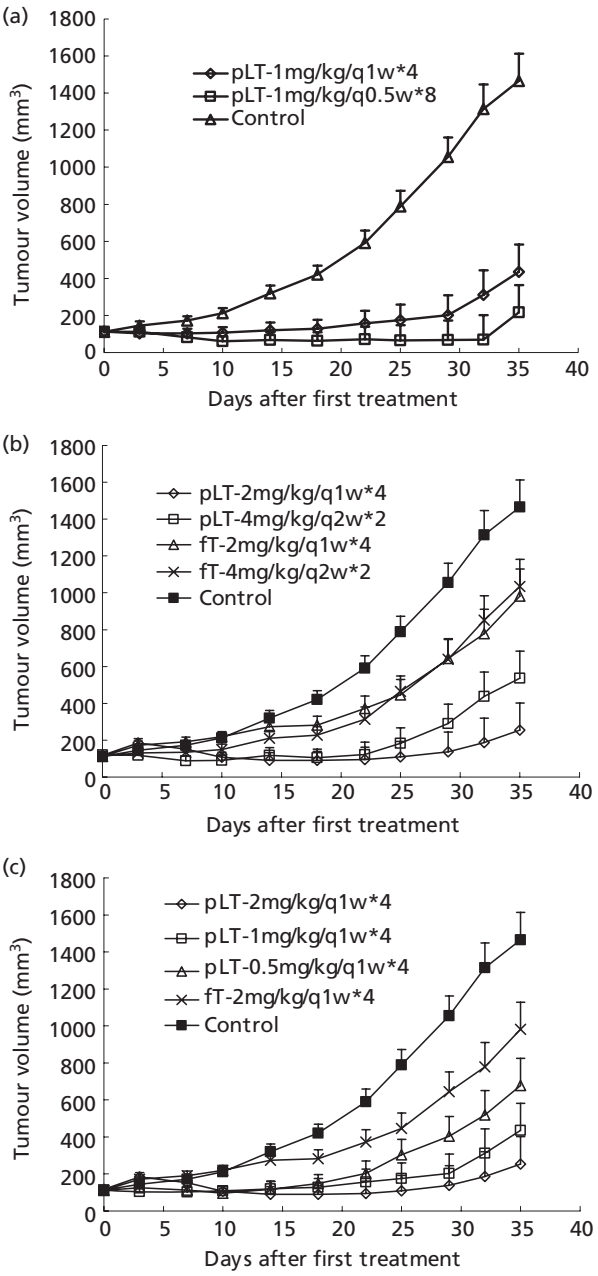

Figure 6 Antitumour effect of liposomal (low polyethylene glycol formulation) and free topotecan in H446 human lung carcinoma bearing male Nu/Nu nude mice. When the mean tumour volume reached to ~110 mm^3, topotecan formulations were injected into mice at different dose schedules (n = 9). liposomal topotecan: 2 mg/kg/q1 weeks × 4 (pLT-2mg/kg/q1w*4), 1 mg/kg/q1 weeks × 4 (pLT-1mg/kg/q1w*4), 0.5 mg/kg/q1 weeks × 4 (pLT-0.5mg/kg/q1w*4), 4 mg/kg/q2 weeks × 2 (pLT-4mg/kg/q2w*2) or 1 mg/kg/q0.5 weeks × 8 (pLT-1mg/kg/q0.5w*8); free topotecan: 2 mg/kg/q1 weeks × 4 (fT-2mg/kg/q1w*4) or 4 mg/kg/q2 weeks × 2 (fT-4mg/kg/q2w*2); control solution: an isotonic sucrose-histidine solution.

grafting density was administrated to beagle dogs at 3.2, 6.4 or 12.8 mg/m^2. Compared with other species, dog is sensitive to skin toxicity induced by PEGylated liposomes containing chemotherapeutic drugs. Previous studies revealed that when PEGylated liposomal doxorubicin and mitoxantrone were injected into dog, skin toxicity could be observed within one to two weeks after administration.[18–20] However, even at the high dose level that could induce animal death, no skin-related toxicity could be observed. Based on our observation, the toxicity of PEGylated liposomal topotecan in dog included significant white blood cell decrease, gastrointestinal mucosa necrosis, acute hepatic injury revealed by increased alanine aminotransferase and aspartate aminotransferase, of which gastrointestinal mucosa necrosis might have been the main cause of death of the dog administered 12.8 mg/m^2. To prevent animal death, the standard PEG formulation was administrated at 6.4 mg/m^2. Interestingly, at this dose level the high PEG formulation could not induce visible skin toxicity either.

Similarly, in mice no skin toxicity could be found. The maximum tolerated dose level for both formulations was similar with a value of 20 mg/kg in Kunming mice and increasing the dose level could induce the death of animals within one week after treatment initiation. The main toxicity was gastrointestinal mucosa necrosis.

Plasma pharmacokinetics and biodistribution

The encapsulation of topotecan into liposomes could significantly alter its plasma pharmacokinetics (Table 1 and Figure 2). In comparison with free topotecan, low PEG liposomal formulations were cleared at a slow rate (10–12 ml/h/kg) and had a small distribution volume (39–49 ml/kg). The area-under-curve (*AUC*) and mean retention time (*MRT*) considerably increased following the administration of liposomal topotecan. For example, at a dose level of 4 mg/kg, the values for *AUC* and *MRT* of the liposomal group were 414.5 ± 62.3 mg/l·h and 4.2 ± 0.5 h, respectively, which were greater than those of free topotecan. Surprisingly, the clearance of liposomal topotecan followed linear kinetics. Namely, *AUC* and the maximum concentration (C_{max}) linearly increased with increasing dose level and other kinetic parameters had almost no changes over the dose range of 0.5–4 mg/kg.

The clearance kinetics of different PEGylated liposomal formulations was compared at 2 mg/kg. As shown in Figure 2c, the pharmacokinetic profiles of both formulations were similar. The standard PEG formulation was also rapidly cleared from the circulation with a half-life of ~ 2 h. The short half-life of the drug might have been associated with the rapid drug leakage rate and the PEG grafting density had almost no effect on the plasma retention time of the drug.

Table 1 Pharmacokinetic parameters for liposomal (low polyethylene glycol formulation) and free topotecan formulations

	Formulations				
	Liposomal topotecan				Free topotecan
Parameters	0.5 mg/kg	1 mg/kg	2 mg/kg	4 mg/kg	4 mg/kg
$AUC_{(0\text{-}t)}$ (mg/l h)	41.829 ± 7.911	94.801 ± 28.635	210.158 ± 45.094	414.524 ± 62.348	2.403 ± 1.166
$AUC_{(0\text{-}\infty)}$ (mg/l h)	42.220 ± 7.847	95.151 ± 28.851	210.766 ± 45.403	418.020 ± 62.433	2.447 ± 1.152
$MRT_{(0\text{-}t)}$ (h)	3.024 ± 0.383	3.604 ± 0.693	3.630 ± 0.406	4.227 ± 0.533	1.396 ± 0.991
$MRT_{(0\text{-}\infty)}$ (h)	3.157 ± 0.266	3.766 ± 0.891	3.700 ± 0.429	4.343 ± 0.589	1.592 ± 1.034
CL (l/h/kg)	0.012 ± 0.003	0.012 ± 0.003	0.010 ± 0.003	0.010 ± 0.001	1.848 ± 0.56
Vd (l/kg)	0.045 ± 0.013	0.048 ± 0.012	0.039 ± 0.004	0.049 ± 0.014	3.007 ± 1.181
C_{max} (mg/l)	18.241 ± 5.282	29.075 ± 3.207	62.402 ± 8.059	103.796 ± 16.018	3.939 ± 1.898

$AUC_{(0\text{-}t)}$, area under curve from time 0 to t; $AUC_{(0\text{-}\infty)}$, area under curve from time 0 to infinite; $MRT_{(0\text{-}t)}$, mean retention time from time 0 to t; $MRT_{(0\text{-}\infty)}$, mean retention time from 0 to infinite; *CL*, clearance; *Vd*, volume of distribution; C_{max}, the peak plasma concentration of the drug.

Table 2 Antitumour efficacy of liposome-entrapped (low polyethylene glycol formulation) and free topotecan formulations against L1210 leukaemia cell line in BDF1 mice

Treatment group	Dose level (mg/kg)	Dosage administration day	No. of survivors (day 60)	Survival time (day)[a]		% ILS[b]	L/F[b]
				Mean	Median		
Control	n.a.	1	0/10	8.9 ± 0.7	8.0		
Topotecan liposomes	8	1	1/10	20.9 ± 4.1	16.0	134.8	1.45
	4	1,8,15	8/10	56.0 ± 2.8	n.a.	529.2	n.a.
	2	1,8,15	8/10	54.7 ± 3.4	n.a.	514.6	
Free topotecan	8	1	0/10	11.7 ± 0.6	11.0 ± 0.3	31.5	
	4	1,8,15	0/10	13.3 ± 1.2	11.0 ± 0.3	23.6	

Mice were inoculated with 5×10^5 cells intravenously on day 0 and treated on day 1. [a]To calculate mean and median survival time, survivors after 60 days were assigned survival times of 60 days. [b]Values for ILS (increased life span) and liposomal/free (L/F) were calculated using median survival data. n.a., not applicable.

Topotecan concentrations in liver and tumour were determined in Lewis lung cancer bearing mice. Compared with free topotecan, the low PEG liposomal formulation preferentially accumulated into tumour, instead of normal tissues (Figure 3). The tumour *AUC* value in liposomal topotecan-treated animals was ~22-fold that of mice treated with the same dose of free topotecan. In contrast, the liver *AUC* value increased 8-fold following the injection of the low PEG liposomal formulation. Both formulations could rapidly accumulate into liver and tumour, but the liposomal formulation was cleared from tissues at a slow rate relative to the conventional formulation. The clearance half-lives of the liposomal formulation in liver and tumour were 3.6 and 5.2 h, respectively.

Antineoplastic activity

Intravenous injection of L1210 leukaemia cells could induce the rapid deposition of cancer cells into liver and thus establish a liver metastasis model. The tumour was uniformly lethal within one to two weeks without treatment. In our study, the control group receiving no drug treatment rapidly died, with a median survival time of ~8.0 days. All the treatments were effective relative to the control solution ($P < 0.05$) and when the same dose schedules were used, the liposomal formulation was more therapeutically active than free topotecan ($P < 0.05$). Surprisingly, it was found that smaller split doses of liposomal topotecan exhibited enhanced efficacy compared with a larger dose, even when the overall dose intensity of small split doses was small. As shown in Table 2, repeated injection of liposomal topotecan at a dose level of 2 mg/kg weekly for three times resulted in a median survival time of 54.7 ± 3.4 days, which was more effective than a single dose of 8 mg/kg liposomal topotecan ($P < 0.05$).

Compared with other tumours, Lewis lung cancer is a rapid growth tumour model. When tumour cells were inoculated into C57 mice the tumours rapidly grew to a mean tumour volume of ~0.3 cm^3 and then the treatments were initialized. All the treatments were effective ($P < 0.05$), and free and liposomal topotecan were therapeutically equivalent at a dose level of 8 mg/kg (Figure 4). However, two doses of 4 mg/kg liposomal topotecan administrated every four days were more active than a single dose of 8 mg/kg liposomal topotecan, indicating that smaller split doses were therapeutically advantageous. Moreover, this dose schedule was more active than 8 mg/kg free topotecan administrated as a single dose.

A similar phenomenon was observed in the RM-1 prostate tumour model (Figure 5). When 8 mg/kg liposomal topotecan was administrated as two split doses, it exhibited enhanced activity compared with the same dose intensity of liposomal topotecan formulation administrated as a single dose ($P < 0.05$). The resulting tumour doubling times were 4.89 and 4.20 days, respectively. In the model, with the exception of free topotecan, all the treatments were efficacious relative to the control group ($P < 0.05$).

In the H446 xenograft tumour model, it was found that smaller doses of PEGylated liposomal topotecan administrated more often tended to be superior therapeutically to larger doses given less often for the dose schedules having the same dose intensity. For instance, weekly intravenous administration of PEGylated liposomal topotecan at a dose of 1 mg/kg (q1 weeks × 4) was less therapeutically active than the same dose intensity injected at a dose level of 0.5 mg/kg every half week × 8 (tumour inhibition on day 35: 70.3% vs 85.2%, $P < 0.05$; Figure 6a). Similarly, keeping the dose intensity unchanged, shortening the dose interval from every four weeks × 2 doses to every two weeks × 4 doses resulted in enhanced antineoplastic efficacy (tumour inhibition on day 35: 63.5% vs 82.7%, $P < 0.05$; Figure 6b). The results presented in the same figure indicated that the alteration of dose interval had almost no effect on the therapeutic activity of free topotecan (2 mg/kg/q1 week × 4 vs 4 mg/kg/q2 weeks × 2, $P > 0.05$). Moreover, when liposomal topotecan was administered at the same dose interval, the resulting therapeutic activity exhibited significant dose dependency (every week × 4 doses, from 0.5 to 2 mg/kg; Figure 6c). Statistical analysis revealed that all the treatments were more effective than the control solution ($P < 0.05$) and liposomal topotecan was more active than free topotecan administered at the same dose schedule (Figure 6b).

Discussion

PEGylated liposomes prepared by the modification of vesicles with PEGlipids (e.g. DSPE-PEG) could prolong the circulation time of vesicles and thus increase the targeting efficiency of vesicles.[1] The biophysical and biological properties of this kind of carrier have been carefully investigated in previous studies.[1,2] To date, the extensively used PEGlipid is DSPE-PEG, a synthetic polymer, in which the lipophilic anchor (DSPE) is linked to the hydrophilic polymer PEG via the carbamate linkage. On the vesicles, the grafted polymer might exhibit at least three conformations, including 'interdigitated mushrooms', 'mushrooms' and 'brushes'.[21,22] The PEG grafting density and its molecular weight determine which conformation regime could be formed. Based on previous investigation, if DSPE-PEG polymer with a mean PEG molecular weight of 2000 was used, the grafting density corresponding to different conformations was < 1%, 1–4% and > 4%, respectively.[21,22] Here, the PEG grafting density referred to the molar percent of DSPE-PEG relative to HSPC.

Typically, to evade recognition by the reticulo-endothelial system and increase the circulation time of vesicles, vesicles are modified at a > 4% PEG grafting density.[4,5,23] For instance, in Doxil, a PEGylated liposomal doxorubicin formulation loaded with the ammonium sulfate gradient method, the PEG grafting density was ~8%.[4,5,23] Therefore, the resulting vesicles were coated with PEG polymers exhibiting dense 'brush' conformation, which could considerably prevent clearance by the RES. However, because Doxil is a slow release formulation, the long circulation time resulted in the accumulation of doxorubicin in the skin, thus inducing a side effect called hand-foot syndrome.[4]

In contrast, the rapid release non-PEGylated doxorubicin liposomal formulation, Myocet, could not induce this kind of side effect in clinical practice.[6,7,14] Nevertheless, Myocet is provided as a complicated three-vial system, which includes empty liposomes, buffer and doxorubicin HCl.[14] Before clinical use, the drug is loaded into liposomes by the clinicians, which might increase the risk of contamination by bacteria thus limiting its clinical application.

In this study, a liposomal topotecan formulation coated with ~3% DSPE-PEG was prepared and its plasma pharmacokinetics were investigated. Despite the fact that the pharmacokinetics of high DSPE-PEG formulations have been investigated extensively, there are almost no reports concerning the pharmacokinetics of low DSPE-PEG formulations.

Previous studies demonstrated that Doxil exhibited one or two phase plasma concentration–time profiles following intravenous injection and the clearance kinetics were linear at low doses.[4] Linear kinetics are helpful for the prediction of plasma concentrations and of toxicity and safety profiles when the dose levels change.

Interestingly, the clearance of the low DSPE-PEG formulation in rat also followed linear kinetics. How to explain this? The characteristics of the vesicles must be taken into account. In our formulation, the PEG grafting density was ~3%. Quick calculation reveals that at this PEG density, the vesicles could be completely covered by the PEG polymer exhibiting mushroom conformation instead of brush conformation. Perhaps, the complete coverage of vesicles with PEG molecules exhibiting mushroom conformation could also prevent the adherence by opsonins and evade the recognition by the RES to a certain degree, resulting in the slow clearance of vesicles by the RES and linear kinetics.

The half-life of the drug for both liposomal formulations (low and high PEG formulations) was short compared with that of Doxil, with a value of ~2 h. The short half-life might be associated with the rapid drug release rate. Unlike doxorubicin, topotecan cannot form a gel precipitate with sulfate,

so it is easily leaked from vesicles. It is well-known that the half-life of liposome-entrapped drugs is determined by the clearance of vesicles and drug release rate. In our study, drug release rate might have played a predominant role in the clearance kinetics of liposomal topotecan, thus resulting in the similar pharmacokinetic profiles for the formulations with different PEG density.

As pointed out in the Introduction, only when cytotoxic drugs are stably encapsulated into vesicles and vesicles circulate long enough, could hand-foot syndrome be induced by drug-containing liposomes accumulated in the skin. If only empty vesicles, not drug-containing vesicles, could accumulate in the skin then no hand-foot syndrome could be induced. Beagle dog is very sensitive to the skin toxicity induced by the PEGylated liposomes. However, in our study no such side effect could be observed, which was in agreement with the results from pharmacokinetic studies.

The vesicles could accumulate rapidly into the tumour as revealed by biodistribution studies. One hour after intravenous injection, the drug concentration in the tumour reached peak concentration and then the concentration gradually decreased with a clearance half-life of ~5.2 h. This data could be used to explain why smaller split doses were more effective than larger doses while maintaining equivalent dose intensity.

Tumour cells are rapid proliferation cells. Like other cells, the cell cycle of tumour cells can be divided into four distinct phases: G1 phase, S phase, G2 phase and M phase. During S phase, DNA is actively synthesized and the amount of DNA in the cell can be effectively doubled when the phase is complete. Topotecan could bind to topoisomerase I-DNA complex and thus induce double-strand DNA damage during DNA synthesis. Accordingly, topotecan is a cell cycle-specific (S phase) drug.[24,25]

Two factors determine whether topotecan could exert its cytotoxicity. One is the topotecan concentration in the tumour, which must be higher than the minimum inhibition concentration of the tumour cells; and the other is how many tumour cells are in the S phase. Based on our observations, the tumour doubling time of Lewis lung cancer in C57 mice was ~3.8 days (control group, untreated), which was much larger than the clearance half-life of topotecan in tumour (~5.2 h). Therefore, some of the tumor cells might not be in the S phase when they are exposed to the high concentrations of topotecan and thus could not be effectively killed by topotecan.

When a large dose was administrated, the drug could rapidly accumulate into the tumour and then was rapidly cleared from the tumour. The bioavailability of the drug might not be as high as that of the small split doses. The modification of the dose schedule by the use of small split doses instead of large doses might prolong the exposure time of tumour cells in the S phase, thus leading to enhanced antitumour efficacy.

Our observation was considerably different to previous studies. Gabizon *et al.*[26] investigated the dose-dependency of the therapeutic efficacy of Doxil in murine models and they observed a trend to superior therapeutic efficacy for treatments based on larger doses as compared with smaller split doses. Here, the differences in both formulations must be considered. First, doxorubicin is not a cell cycle-specific drug; second, Doxil is a slow release formulation and it is slowly cleared from the tumours.[4,23,26]

We did not determine topotecan concentrations in the liver of tumour bearing mice (L1210 liver metastasis model), but the mechanism might be similar since liposomal topotecan was rapidly cleared from liver in healthy mice.

Conclusions

Topotecan could be effectively loaded into low DSPE-PEG vesicles using the ammonium sulfate method. The clearance kinetics of the resulting vesicles from plasma was linear in the rat. Following intravenous injection, topotecan-containing vesicles could rapidly accumulate into the tumour and then were cleared from the tumour with a half-life of ~5 h, which could explain why small split doses were more effective. Due to its short circulation half-life the formulation could not induce skin toxicity. Our data revealed that low DSPE-PEG formulations are worthy of further investigation, and the dose schedule of liposomal drugs should be adjusted in accordance with the biophysical and biological properties of the formulations to achieve the optimal therapeutic efficacy.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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