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Research paper

A novel method to load topotecan into liposomes driven by a transmembrane NH_4EDTA gradient

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ABSTRACT

Antitumor drugs not only cause cytocidal effect on cancer cells, but also damage on normal healthy tissues, resulting in side effects. Liposome encapsulation can result in reduced systematic distribution due to the enhanced permeability and retention (EPR) effect, accompanied by drug accumulation in liver, spleen, and other immune organs, which can cause damage to those organs. It has been demonstrated that EDTA, frequently used as a chelator, possesses a synergistic antitumor effect. Indeed, our previous study showed that EDTA could reduce the toxicity of anthracyclines to the heart and immune organs. In this study, we intended to encapsulate topotecan within liposome adopting transmembrane NH_4EDTA gradient in order to increase the antitumor activity and decrease the toxicity against normal immune organs. Regarding the encapsulation efficiency of topotecan liposomes, both the pH value of the buffer and the cholesterol content showed significant effects on encapsulation and drug retention. Liposome encapsulation dramatically increased the antitumor activity of topotecan compared to free drug ($p < 0.05$), while similar efficacy was obtained from liposomes prepared by a NH_4EDTA gradient or a $(NH_4)_2SO_4$ gradient (tumor inhibition ratios were 85.6% and 84.1%, respectively). However, a significant decrease in toxicity against the immune organs was found in liposomes prepared by a NH_4EDTA gradient compared to those prepared by a $(NH_4)_2SO_4$ gradient. These results suggest the superiority of the proposed gradient for topotecan encapsulation in decreasing its toxicity on immune systems.

1. Introduction

Topotecan, a hydrosoluble semi-synthetic derivative of camptothecin [1], is one of the two camptothecin analogues currently approved for clinical application by FDA [2]. It is being clinically used to treat refractory ovarian cancer and/or small-cell lung cancer, which are resistant to conventional chemotherapeutic agents [3,4]. Furthermore, the combination of topotecan with other standard chemotherapeutic agents is increasingly being used for improved therapy [5]. Similar to camptothecin and other derivatives, topotecan specifically inhibits the activity of topoisomerase I by stabilizing the topoisomerase I–DNA complex, resulting in lethal DNA strand breaks [6]. However, despite the excellent antineoplastic activity of topotecan, its severe side effects hamper its clinical use. Preclinical studies showed greater antitumor efficacy with prolonged low-dose exposure to topotecan, and in animal models, low-dose prolonged exposure resulted in less toxicity [7,8]. Therefore, increased antitumor efficacy and decreased toxicity could be achieved by prolonged exposure of tumor tissues to the drug. Encapsulation of the drug within liposomes has been reported to prolong the exposure time [9].

Liposomes have been used for drug delivery during the past three decades. And numerous studies have shown that liposome encapsulation of anticancer agents can alter tissue disposition [10,11], reduce blood clearance rate [12], decrease systematic toxicity, and enhance antitumor activity [13,14]. In case of topotecan, liposomes could further provide protection against hydrolysis of the α-hydroxy-δ-lactone ring due to the acidic interior of the carrier after loading using electrochemical gradients [6]. Additionally, the closed lactone ring was reported to be structurally important for both passive diffusion of topotecan into cancer cells [15,16] and successful interaction with the topoisomerase target [17]. Although the EPR effect of liposomes has been reported to enhance antitumor efficacy and reduce systematic toxicity, only a few studies have been focused on the damage caused by antitumor drug-loaded liposomes on the immune organs due to the uptake of liposomes by phagocytes of these organs. It is well known that the thymus is a central immune organ, in which lymphocytes

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mature, multiply, and become T cells [18]. The spleen is the largest lymphatic organ in the body and provides lymphocytes and a source of plasma cells and hence antibodies for the cellular and humoral-specific immune defenses [19]. Therefore, all of them are of vital importance to maintain normal life activities.

EDTA is a frequently used chelating agent. The use of EDTA is the standard FDA approved treatment for lead, mercury, aluminum, and cadmium poisoning [20]. Previous reports have indicated that EDTA plays an important role in heart disease and cancers [21–23]. Moreover, a study by Walter Blumer and Elmer Cranton [24] showed that the mortality from cancer was reduced 90% during an 18-year follow-up of 59 patients treated with calcium EDTA. Meanwhile, the most popular metabolic emergency of tumor patients is hypercalcinemia, and numerous studies have demonstrated the complication of hypercalcinemia in general lymphadenomatosis of bones, lymphoma, mammary adenocarcinoma, and several other tumors [25]. Therefore, when a transmembrane NH_4EDTA gradient is used to load antitumor drugs into liposomes, the intraliposomal EDTA could chelate extra calcium, resulting in improved quality of life for the patient. Additionally, our previous study showed that liposomes prepared by a transmembrane NH_4EDTA gradient significantly decreased the toxicity of cytotoxic drugs on the immune system compared with liposomes prepared by a $(NH_4)_2SO_4$ gradient, regardless of encapsulation of doxorubicin, epirubicin, or vinorelbine. Additionally, the use of EDTA in the formulations might chelate the residual metal ions of liposomes and avoid the catalytic oxidation effect of metal ions on phospholipids, so as to increase the stability of liposomal preparations.

Therefore, in this study, liposomes prepared by a transmembrane NH_4EDTA gradient were used to encapsulate topotecan with the expectation of enhancing therapeutical efficacy and decreasing the liposome-related damage to the immune organs. Concerning toxicity analysis, a locomotor activity boxes experiment was used to estimate the status of mice, which could directly reflect the behavior of each mouse by the amount of activity performed within 5 min rather than described by some vague concepts as before. Also, our study is the first time to adopt a locomotor activity boxes experiment for evaluating the status of animals in the field of liposomal drug delivery system. As with some other anticancer drugs such as doxorubicin, epirubicin, and vinorelbine, our results showed that compared with liposomes prepared by a $(NH_4)_2SO_4$ gradient, liposomes prepared by a transmembrane NH_4EDTA gradient had a decreased level of toxicity to the immune system. This technique may be an important alternative to other methods offering comparative therapeutical efficacy but reduced toxicity.

2. Materials and methods

2.1. Materials

Hydrogenated soy phosphatidylcholine (HSPC, MW 785) was obtained from Lucas Meyer (Düsseldorf, Germany). Cholesterol (CH) was supplied by Nanjing Xinbai Pharmaceutical Co. Ltd. (Nanjing, China). Topotecan HCl (TPT, solubility of topotecan and topotecan HCl in water is <1 mg/mL and >30 mg/mL, respectively) was provided by Chengdu Tianyuan natural product Co. Ltd. (Chengdu, China). 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] ($mPEG_{2000}$-DSPE) was purchased from J&K Chemical Ltd. (Beijing, China). Anion- and cation-exchange resins were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Sephadex G-50 (medium) was obtained from the Sigma Chemical Company (St. Louis, MO, USA). All other chemicals were of analytical grade.

2.2. Animals

Kunming male mice weighing 18–22 g and male beagle dogs weighing 8–10 kg were purchased from the Laboratory Animal Center of Shenyang Pharmaceutical University (Shenyang, China). Principles in good laboratory animal care were followed, and animal experimentation was in compliance with the Guidelines for the Care and Use of Laboratory Animals at Shenyang Pharmaceutical University.

2.3. Preparation of liposomes

An ammonium salt solution of EDTA was prepared as follows: 1.465, 2.198, 2.930, or 3.663 g ethylenediaminotetraacetic acid (EDTA) was weighed and mixed with 45 mL of distilled water with the subsequent dropwise addition of ammonia water at 80 °C to the agitated suspension until formation of a clear solution. Then, the solution was allowed to cool at room temperature, and ammonia water was added to produce a solution with pH values of 5.0, 5.5, 6.5, or 7.0. Finally, the solution was transferred to the volumetric flask and diluted with water to 50 mL to obtain the buffers with different EDTA concentrations or different pH values.

Liposomes were prepared by a modified ethanol injection method (see Table 1). Briefly, a lipid mixture containing HSPC/CH/$mPEG_{2000}$-DSPE (3/1/1, W/W) was dissolved in ethanol, and the solvent was then removed at 65 °C. The resulting lipid film was hydrated with NH_4EDTA solution under mechanical agitation. Liposomes consisted with HSPC/CH/$mPEG_{2000}$-DSPE (3/0/1, W/W) or HSPC/CH/$mPEG_{2000}$-DSPE (3/0.5/1, W/W) were also prepared to determine the influence of the cholesterol content on TPT encapsulation. Additionally, for the *in vitro* release test and animal experiments, liposomes containing internal solutions of 200 mM $(NH_4)_2SO_4$ (pH 5.5) were prepared.

To reduce liposome size, the large liposome suspension initially produced was sonicated using a JY92-2D Vibra-cell probe sonicator (Ningbo Xinzhi Biological Technology Co. Ltd., China) equipped with a tapered tip, for at least additional six-minute cycle (400 w) followed by a two-minute cycle (200 w). In all cases, the initially turbid liposomal suspension was well clarified after sonication. Following sonication, the suspensions were then filtered through 0.8-, 0.45-, and 0.22-μm filter membranes at 25 °C, respectively, to remove large particles. The mean diameters and size distribution of the liposomes were determined in purified water at 23 °C using a NICOMP 380 HPL submicron particle analyzer (Particle Sizing System, CA, USA) operated at 632.8 nm.

2.4. Preparation of the ammonium gradient for drug encapsulation

Liposomes were passed through an ion-exchange resin column (Anion-exchange resin: Cation-exchange resin = 2:1, V/V) equilibrated with purified water to replace the extraliposomal solution, and final phospholipid concentrations of the eluted gradient liposomes were ~20 mg/mL. Subsequently, TPT solution (2 mg/mL) was added to the liposomal dispersion to achieve a drug-to-lipid ratio of 0.1 (W/W). The loading process was carried out at 50 °C for 10 min and then quickly cooled in an ice/water bath (0–2 °C) for 5 min. To determine the kinetics of drug encapsulation, 0.4 mL of liposomal samples was taken after loading for 1, 5, 10, 20, 40, or 60 min. Furthermore, in order to determine the influence of the external pH on TPT encapsulation, 0.2 mL of the above-mentioned gradient liposomes was mixed with 0.2 mL histidine buffer (100 mM, pH of 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, and 9.5, respectively) and 0.2 mL TPT solution (2 mg/mL), and the mixture was incubated at 50 °C for 10 min.

Table 1
Physico-chemical parameters of the liposomes used for the study.

Formulation	Liposome composition	Salt gradient	Particle size before loading (nm) (±SD)	Particle size after loading (nm) (±SD)	Drug-to-lipid ratio (W/W)
NH_4EDTA-L	HSPC/CH/mPEG$_{2000}$-DSPE (3/1/1, W/W)	NH_4EDTA	86.6 ± 3.9	84.8 ± 3.8	0.1
$(NH_4)_2SO_4$-L	HSPC/CH/mPEG$_{2000}$-DSPE (3/1/1, W/W)	$(NH_4)_2SO_4$	86.7 ± 4.5	83.2 ± 1.5	0.1

Data are presented as mean values from three repeated experiments.

2.5. Determination of encapsulation efficiency (EE)

The separation of liposomes from free TPT was performed by size exclusion chromatography on a Sephadex G-50 mini-column (10 × 70 mm). Separation of 100 μL liposomes from nonencapsulated TPT was carried out by elution with 5% glucose solution. TPT concentration was determined by a UV-spectrophotometer (Uvikon 756MC, The Third Analysis Instrument Factory of Shanghai, China) at 430 nm after lysis of the liposomes with 70% (v/v) ethanol containing 0.1 mol/L NaOH.

2.6. Release assay in vitro for TPT

In the release assay, TPT liposomes or solution was placed in dialysis tubing with a molecular weight cutoff of 10 kDa and dialyzed against 100 mL of PBS (pH 7.4) at 37 °C. At indicated time points, aliquots were withdrawn and the fluorescence intensity of the samples was measured (Ex = 413 nm, Em = 533 nm) using a 650-60 fluorescence spectrophotometer (HITACHI, Japan).

2.7. Antitumor activity and acute toxicity test

A xenograft mouse model of human liver cancer (H_{22}) was used for the analysis of antitumor activity. Briefly, tumors were established by injecting H_{22} tumor cells (Provided by the Cancer Institute of Liaoning Province, China) subcutaneously at the right axillary flank of 6–8 weeks old, Kunming male mice (Laboratory Animal Center of Shenyang Pharmaceutical University, Shenyang, China) on day 0. Treatments were initiated as multiple dosing on days 4, 7, and 10. For the treatment groups ($n = 6$), drug dosage was 5 mg/kg. Control animals received injections of sterile saline. Mice were weighed on the day of tumor injection, and their weights were recorded every two days until the first death in the control group. When the mortality of the control group was >20%, the animals were sacrificed, and tumor mass, spleen mass, and thymus mass were measured separately to evaluate the antitumor activity and toxicity of different formulations.

For acute toxicity test, thirty Kunming male mice from the Laboratory Animal Center, Shenyang Pharmaceutical University (Shenyang, China), weighing 18–22 g were used and randomly divided into three groups with each group of 10. All animals were acclimatized in wire cages in a 12 h light–dark cycle for a minimum of 2 days before starting the experiment to allow them to adjust to the new environment. During this period, mice had free access to food and water. Then, three groups were treated with TPT solution, $(NH_4)_2SO_4$-L, and NH_4EDTA-L, respectively, at a dose of 35 mg/kg. During the experiment, the weight loss and other signs of stress/toxicity of mice were monitored for a period of 4 days. And the mortality in each group was recorded. Meanwhile, the status of mice was estimated by a locomotor activity boxes experiment (Shanghai Zhongsheng Co. Ltd.). Briefly, mice were put into the locomotor activity boxes, and the amount of activity of each mouse within 5 min was recorded automatically by the instrument, which could be used to reflect the mobility of mice. After 4 days, all remaining animals were sacrificed, and necropsies were conducted to identify any additional drug toxicities. Survival analysis was performed using GraphPad Prism software (5.0 versions).

2.8. Pharmacokinetic studies in beagle dogs

Beagle dogs were used as the animal model for pharmacokinetic studies due to their large volume of circulating blood and relatively small individual differences. Free TPT, NH_4EDTA-L, and $(NH_4)_2SO_4$-L were injected i.v. into beagle dogs at a dose of 0.5 mg/kg TPT, respectively, and the plasma elimination of the drug was determined over a 24-h time course. Total TPT was determined (Ex = 380 nm, Em = 531 nm) using a 650-60 fluorescence spectrophotometer (HITACHI, Japan), and quantitation of the lactone form of TPT was performed by HPLC analysis. The pharmacokinetic parameters were calculated using DAS 2.1.1 software (the net for drug evaluation of China).

2.9. Statistical analysis

Statistical comparisons were performed using a One-Way ANOVA assay or univariate test with SPSS 16.0 software. p values lower than 0.05 were considered statistically significant.

3. Results

3.1. Effect of the incubation temperature on the encapsulation efficiency (EE)

As seen in Fig. 1, appreciable drug uptake (78%) was observed at 40 °C, and this loading course was found to be temperature-dependent, with the maximum encapsulation efficiency achieved at 50 °C (85%, $p < 0.05$ vs. the encapsulation efficiency at 40 °C). However, when the temperature was >50 °C, the entrapment efficiency decreased slightly. This may be due to the high permeability of the bilayer and increased molecular motion of drug at high temperatures.

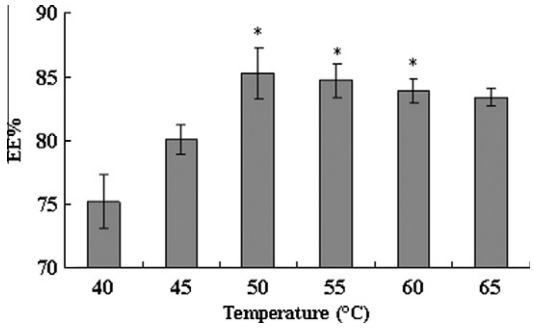

Fig. 1. Effect of incubation temperature on the drug loading efficiency of HSPC/CH/mPEG$_{2000}$-DSPE (3/1/1, W/W) liposomes. The lipid formulations were prepared with a drug/lipid ratio of 0.1 (W/W). The lipid film was hydrated with 200 mM NH_4EDTA solution adjusted to pH 7.0. Liposomes were incubated with TPT for 5 min at different temperatures. p Values apply to differences between the encapsulation efficiencies of liposomal TPT incubated at 40 °C and that at other temperatures, $^*p < 0.05$. Data points represent the mean encapsulation efficiencies of three replicate experiments, and the error bars indicate the standard deviation.

3.2. Effect of the internal pH on TPT encapsulation

HSPC/CH/mPEG$_{2000}$-DSPE (3/1/1, W/W) liposomes were prepared with intraliposomal NH_4EDTA buffer (200 mM) with various pH values (pH 5.0, 5.5, 6.5, and 7.0). Drug loading was carried out at 50 °C with a drug-to-lipid ratio of 0.1(W/W). As shown in Fig. 2, TPT encapsulation and retention were found to be pH-dependent with the faster rate of loading being observed at higher pH values and better retention ability at lower pH values. For the systems with pH values of 6.5 and 7.0, the maximum encapsulation efficiencies were achieved at the first 1 min (89.6% and 84.3%, respectively). However, the EE was not stable, and the loading efficiencies measured at 60 min decreased for both formulations (83.7% and 77.1%, respectively). For the systems with pH values of 5.0 and 5.5, loading was relatively slow (with the maximum EE achieved at 20 min for pH values of 5.0 and 5 min for pH 5.5, respectively). Also, for these two formulations, drug retention was more satisfactory, and the EE was >87% during the time course of 20–60 min. Interestingly, the loading capacity for the system with a pH of 5.0 was lower than that with a pH of 5.5, which may be due to the insufficient ammonium gradient.

3.3. Effect of the NH_4EDTA concentration on the encapsulation efficiency (EE)

Liposomes were prepared by hydrating the lipid film with various buffers containing 100, 150, 200, or 250 mM NH_4EDTA (pH was adjusted to 5.5 with $NH_3{\cdot}H_2O$). As depicted in Fig. 3, the maximum encapsulation efficiency of liposomes prepared with 200 mM NH_4EDTA was significantly higher than the efficiency observed using 250 mM NH_4EDTA ($p < 0.05$). And there was clearly more drug uptake at 1 min for liposomes prepared with 200 mM NH_4EDTA (78.7%) than for other three formulations (55.4%, 63.7%, and 47.1% for 100, 150, and 250 mM NH_4EDTA liposomes, respectively), indicating the faster loading rate for liposomes prepared with 200 mM NH_4EDTA than other three formulations. Therefore, 200 mM was regarded as the optimal concentration of NH_4EDTA.

3.4. Effect of cholesterol content on entrapment efficiency

Cholesterol affects not only the physical properties of bilayer such as fluidity, stability, and permeability, but also the retention characteristics of some relatively hydrophobic drugs due to the interaction between cholesterol and drug [26,27]. Therefore, we investigated the effect of cholesterol content on the encapsulation and retention characteristics of topotecan intraliposomes. As shown in Fig. 4, cholesterol significantly affected loading efficiency and drug retention. There was nearly no drug encapsulated into liposomes with a cholesterol content of 0 mol% (with EE <2% during the time course of 1–60 min), and as the cholesterol content increased, the entrapment efficiency also increased, with >90% efficiency achieved when cholesterol content was 38.5 mol% (i.e., HSPC/CH/mPEG$_{2000}$-DSPE = 3/1/1, W/W).

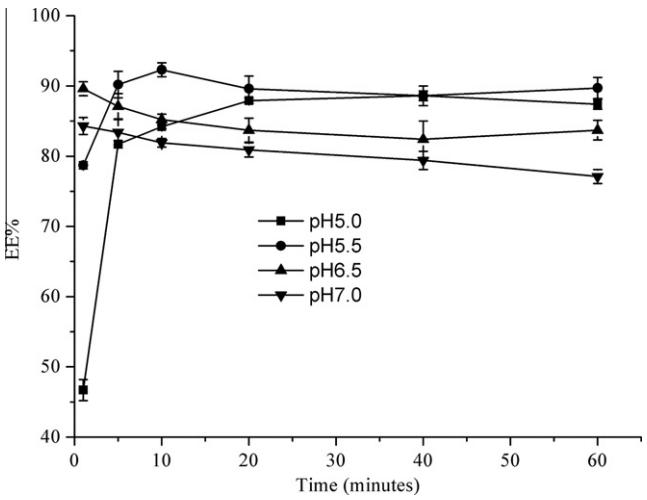

Fig. 2. Loading of TPT into HSPC/CH/mPEG$_{2000}$-DSPE (3/1/1, W/W) liposomes prepared with 200 mM NH_4EDTA buffers of different pH values. The results represent the mean encapsulation efficiencies determined from three separate experiments, and the error bars indicate the standard deviation.

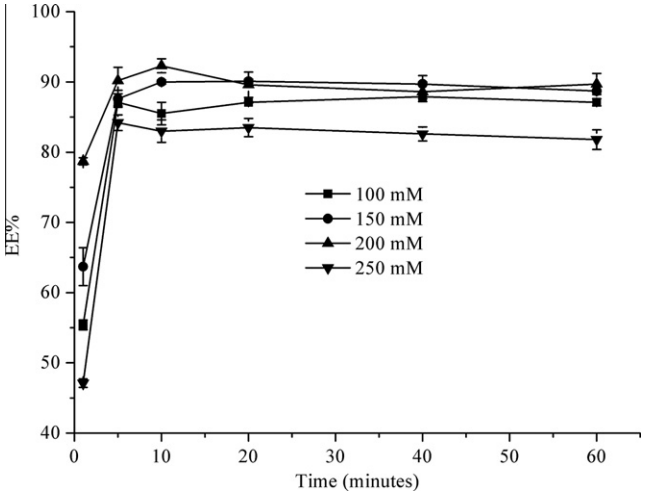

Fig. 3. Encapsulation efficiency (EE) of TPT in HSPC/CH/mPEG$_{2000}$-DSPE (3/1/1, W/W) liposomes by varying the intraliposomal NH_4EDTA concentration (pH 5.5). Data points represent the mean encapsulation efficiencies of three replicate experiments, and the error bars indicate the standard deviation.

3.5. Loading of TPT via NH_4EDTA gradient with different exterior pH values

The extraliposomal solution pH of the buffer had a dramatic effect on loading, with >88% efficiency achieved between pH 3.5

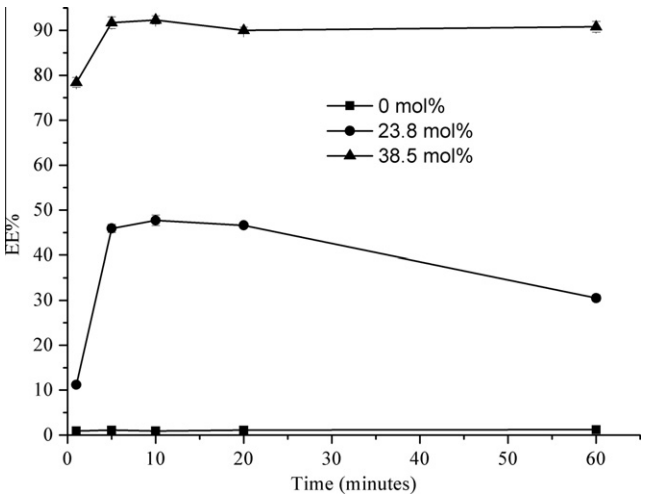

Fig. 4. Encapsulation efficiency of TPT liposomes with different cholesterol contents. Liposomal TPT was prepared at a drug-to-lipid ratio of 0.1 (W/W) with 200 mM NH_4EDTA (pH 5.5). Loading was performed at 50 °C for a period of time. After removal of extraliposomal drug by size exclusion chromatography, the drug of pre- and post-column samples was analyzed and % encapsulation was calculated. The results represent the mean encapsulation efficiencies determined from three separate experiments, and the error bars indicate the standard deviation.

and 7.5, while a substantial reduction in drug entrapment efficiency (69.1 ± 0.82) was observed as the pH was increased above 8.5 (Fig. 5). This may be due to a decreased amount of membrane permeable electroneutral form of TPT present at a pH $\geqslant$8.5 [28]. However, loading was quantitative over a broad range of pH values from 3.5 to 7.5.

3.6. Leakage of TPT liposomes in the presence of PBS

To assess the stability of the TPT-bearing liposomes loaded via transmembrane gradients, two different liposomal preparations were studied. The drug release profile from the TPT-loaded liposomes in the presence of PBS (pH7.4) is shown in Fig. 6. Free TPT could completely permeate through the dialysis tubing at 4 h while there was a small amount of drug leaked from both NH_4EDTA-L and $(NH_4)_2SO_4$-L (leakage of TPT were 6% and 4% at 4 h, respectively, $p < 0.05$ vs. TPT solution). However, to our surprise, drug leakage from NH_4EDTA-L was slightly more rapid than that from $(NH_4)_2SO_4$-L although there was no significant difference ($p > 0.05$) between these two groups during the time course of 24 h.

3.7. Antitumor activity and acute toxicity test

Antitumor efficacy of TPT solution, NH_4EDTA-L, and $(NH_4)_2SO_4$-L was evaluated in a rapidly growing H_{22} human liver cancer xenograft model in mice (Fig. 7, Table 2). Free TPT was well tolerated at the dose given, with a slight increase in animal weights after treatment. In case of the liposomal TPT, weight loss was observed after the second administration in both NH_4EDTA-L and $(NH_4)_2SO_4$-L, especially in $(NH_4)_2SO_4$-L ($p < 0.05$ vs. either NS or TPT solution), in which one drug-related death was observed. In general, liposomal TPT showed activity superior to that of the equivalent dose of free drug ($p < 0.05$); however, a comparative tumor inhibition effect was observed for NH_4EDTA-L and $(NH_4)_2SO_4$-L ($p > 0.05$). Meanwhile, we compared the effect of different TPT formulations on immunity organs of mice using the spleen index and thymus gland index as indicators (weight of spleen or thymus gland/body weight of mouse, mg/10 g). As described in Table 3, when the thymus gland index was used as the indicator, compared with the group treated with NS, no clear thymus gland weight loss was observed in NH_4EDTA-L ($p > 0.05$), but significant thymus gland weight loss was observed in $(NH_4)_2SO_4$-L ($p < 0.05$). When the spleen index was used as the indicator, compared with group treated with either NS or NH_4EDTA-L, significant spleen weight loss was observed in $(NH_4)_2SO_4$-L ($p < 0.05$). These results preliminarily suggested that the toxicity of $(NH_4)_2SO_4$-L was more severe than that of NH_4EDTA-L.

To further investigate the toxicity of these two TPT-loaded liposomes, an acute toxicity test was carried out (Fig. 8). The status of mice was estimated by a locomotor activity boxes experiment before injection, which demonstrated similar activity among three groups ($p > 0.05$). However, at 1 h post-injection, the status of mice

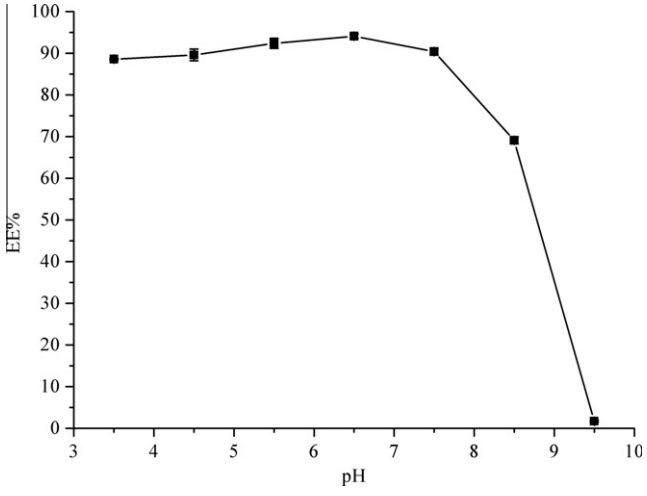

Fig. 5. The drug loading efficiency of HSPC/CH/mPEG$_{2000}$-DSPE (3/1/1, W/W) liposomes as a function of exterior pH values. After removal of extraliposomal solution by ion-exchange resin method, histidine buffer of various pH values was added, and the liposomal TPT was prepared at a drug-to-lipid ratio of 0.1(W/W) with 200 mM NH_4EDTA (pH 5.5). Loading was initiated by incubation at 50 °C for 10 min. Then, the% encapsulation was calculated. Data are shown as mean ± SD, $n = 3$.

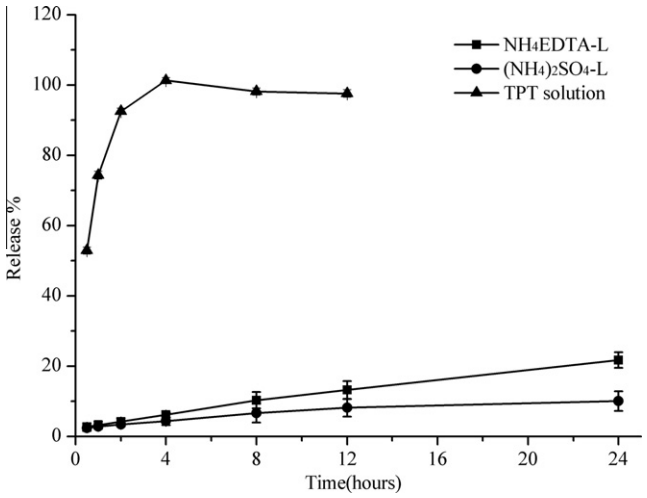

Fig. 6. Release *in vitro* of TPT from HSPC/CH/mPEG$_{2000}$-DSPE (3/1/1, W/W) liposomes using NH_4EDTA gradient and $(NH_4)_2SO_4$ gradient in the presence of PBS (pH 7.4). Drug release was determined *in vitro* as described in the methods, where liposomes were loaded with TPT at a 0.1 drug-to-lipid ratio (W/W). At indicated time points, aliquots were withdrawn and the fluorescence intensity of the samples was measured (Ex = 413 nm, Em = 533 nm). Therefore, the accumulative release was calculated for each formulation. Data are shown as mean ± SD, $n = 3$.

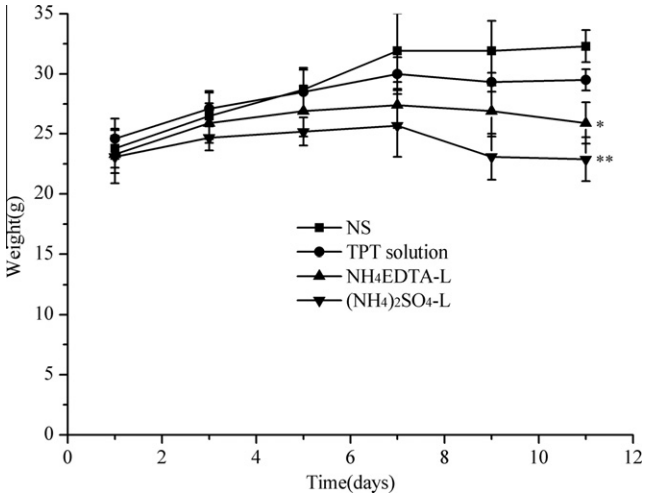

Fig. 7. Body weight change in H_{22}-bearing mice. Different liposomal TPT formulations and free TPT were injected into mice implanted with H_{22} human liver cancer cells at a dose level of 5 mg/kg. Control group was treated with isotonic normal saline. Weights of mice were recorded every 2 days until the first death in the control group. The values were shown as mean ± SD ($n = 6$). *Differences between the groups vs. NS are statistically significant at the $p < 0.05$ level. **Differences between the groups vs. NS are statistically significant at the $p < 0.005$ level.

Table 2
Tumor inhibition effect of different TPT formulations ($n = 6$).

Group	Number of deaths	Survival rate (%)	Tumor inhibition ratio (%)	$p1$[a]	$p2$[b]	$p3$[c]
NS	4	33.3	/	/	<0.05	<0.05
TPT solution	0	100	46.3 ± 25.6	<0.05	/	<0.05
NH_4EDTA-L	0	100	85.6 ± 6.1	<0.01	<0.05	>0.05
$(NH_4)_2SO_4$-L	1	83.3	84.1 ± 7.6	<0.01	<0.05	/

Tumor inhibition ratio% = $(W_N - W_S)/W_N \times 100$.
W_N indicates the average tumor weight of mice in the control group;
W_S indicates the tumor weight of mice in the treated groups.
[a] p Value vs. NS-treated mice.
[b] p Value vs. TPT solution-treated mice.
[c] p Value vs. $(NH_4)_2SO_4$-L-treated mice.

Table 3
Damage produced in immunity organs of H_{22}-bearing mice by different formulations.

Treatment group	Spleen index (mg/g)	Thymus gland index (mg/g)	$p1$[a]	$p2$[b]	$p3$[c]	$p4$[d]	$p5$[e]	$p6$[f]
NS	4.74 ± 1.45	2.49 ± 1.38	/	>0.05	<0.05	/	>0.05	<0.05
TPT solution	3.74 ± 0.83	1.24 ± 0.39	>0.05	/	>0.05	>0.05	/	>0.05
$(NH_4)_2SO_4$-L	2.92 ± 0.49	1.07 ± 0.45	<0.05	>0.05	/	<0.05	>0.05	/
NH_4EDTA-L	3.84 ± 0.80	1.59 ± 0.85	>0.05	>0.05	<0.05	>0.05	>0.05	>0.05

[a] p Value vs. NS-treated mice for spleen index analysis.
[b] p Value vs. TPT solution-treated mice for spleen index analysis.
[c] p Value vs. $(NH_4)_2SO_4$-L-treated mice for spleen index analysis.
[d] p Value vs. NS-treated mice for thymus gland index analysis.
[e] p Value vs. TPT solution-treated mice for thymus gland index analysis.
[f] p Value vs. $(NH_4)_2SO_4$-L-treated mice for thymus gland index analysis.

treated with TPT solution was obviously worse than those of other two groups (locomotor activity test indicated a significant decrease in the amount of activity compared with other two groups, $p < 0.05$), and also, drug-related death was observed in this group. Since then mice in this group began to urinate frequently, and drug was gradually cleared with the urine, leading to recovery within 24 h (locomotor activity test showed no significant difference among these three groups at 24 h, $p > 0.05$). As the observation time increased, the mortality of all these three groups increased with decreased body weight and the survival status of mice (the amount of activity was significantly reduced at 72 h), especially in the group treated with $(NH_4)_2SO_4$-L, which had the most obvious weight loss and the highest death rate, with 80% mortality on the fourth day after administration. When the mouse died, spleen was removed to calculate the spleen index and evaluate the damage of different formulations on the immune system (Table 4). As demonstrated in Table 4, there was a significant difference between the group treated with $(NH_4)_2SO_4$-L and the group treated with either TPT solution or NH_4EDTA-L ($p < 0.05$), indicating greater damage to the immune system by liposomes prepared by the $(NH_4)_2SO_4$ gradient.

Various TPT liposomes and TPT solution were injected into mice implanted with H_{22} human liver cancer cells at a dose level of 5 mg/kg. Control group was treated with isotonic normal saline. When the mortality of the control group was >20%, animals were sacrificed, and spleen mass and thymus mass were measured separately to evaluate the toxicities of different formulations. The values were shown as mean ± SD ($n = 6$).

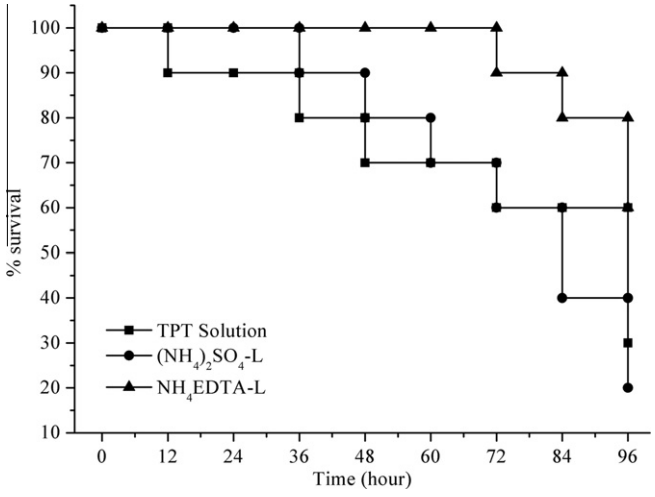

Fig. 8. Acute toxicity studies ($n = 10$). Different TPT formulations were injected into mice via tail veins at a TPT dose of 35 mg/kg. Following administration, mice were monitored daily, the number of deaths was recorded, and survival analysis was performed using GraphPad Prism software (5.0 versions).

3.8. Plasma elimination of liposomal TPT

The mean plasma concentration–time profiles of TPT based on lactone form or total TPT following single i.v. dose (0.5 mg/kg) of different liposomal TPT and free TPT in beagle dogs were shown in Fig. 9. Liposomal encapsulation significantly increased the plasma TPT level and decreased the plasma clearance of TPT ($p < 0.05$). The area under the curve (AUC_0) of NH_4EDTA-L and $(NH_4)_2SO_4$-L were 30-fold and 69-fold of that of free drug based on total TPT measurements, respectively. The plasma clearance rate (Cl) of free TPT, NH_4EDTA-L, and $(NH_4)_2SO_4$-L were 0.390 ± 0.015, 0.019 ± 0.005, and 0.008 ± 0.001 L/h/kg separately.

Table 4
Spleen index analysis of acute toxicity studies ($n = 10$).

Formulation	Spleen index (mg/g)	p Value for spleen index analysis	Compared formulation
TPT solution	2.00 ± 0.48	<0.05	$(NH_4)_2SO_4$-L
NH_4EDTA-L	2.29 ± 0.76	>0.05	TPT solution
$(NH_4)_2SO_4$-L	1.51 ± 0.24	<0.05	NH_4EDTA-L

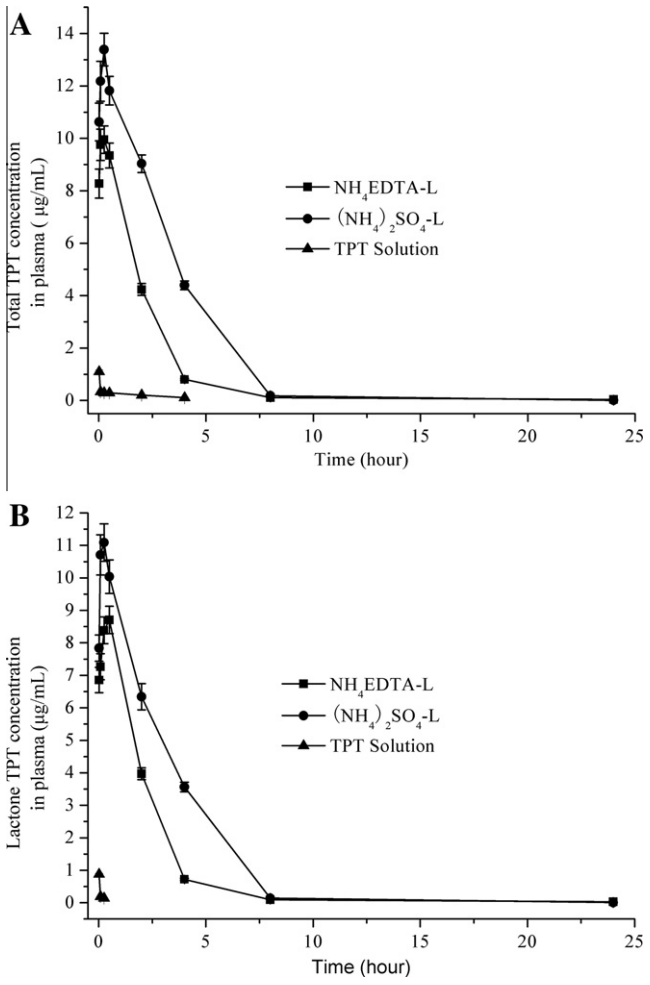

Fig. 9. Concentration–time curve of TPT based on total TPT (A) or lactone TPT (B) measurements after single i.v. dose (0.5 mg/kg) of free TPT and different liposomal TPT. Each value represents mean ± SD of three animals.

The lactone protecting effect *in vivo* was also observed, because the lactone form is reported to be the active species of TPT. For free TPT, the lactone form of the drug in plasma were ~80%, 60%, and 50% at 1 min, 5 min, and 15 min following injection, respectively, and at 30 min after injection, the lactone form of TPT could not be detected in plasma. In contrast, liposomal topotecan is protected as the lactone species. Up to 8 h after injection, ~80% of the topotecan recovered from plasma was in the active lactone form for both NH_4EDTA-L and $(NH_4)_2SO_4$-L.

Interestingly, it is worth noting that in accordance with the *in vitro* release test, the *in vivo* results also demonstrated an unexpectedly rapid drug elimination profile for NH_4EDTA-L, indicating that EDTA was not as successful in stabilizing TPT as compared to that of some anthracyclines such as doxorubicin and idarubicin [27].

4. Discussion

Loading liposomes with TPT via transmembrane NH_4EDTA gradient is a new, potent, and alternative remote-loading method, which takes into account both the efficacy and toxicity of cytotoxic drug-loaded liposomes. Previous studies on TPT liposomes were mainly focused on improving drug retention inside the liposomes, associated with enhanced antitumor efficacy [28–30]. However, it is well known that the cure rate is very low for patients with metastatic disease, and it is difficult to accurately evaluate the therapeutic effect only using cure rate as an index. Moreover, the severe side effects of antitumor drugs also decreased the quality of life of patients. Even when drugs are encapsulated within liposomes, damage of these antitumor drugs to some important organs cannot be ignored due to the accumulation of liposomes into these organs. Therefore, besides guaranteeing the therapeutic efficiency of antitumor drugs, care for patient's quality of life is also of vital importance, especially for patients with advanced cancer. For such patients, survival with a high quality of life is even more meaningful [31,32].

Therefore, in this study, liposomes prepared by a transmembrane NH_4EDTA gradient were employed to encapsulate TPT with the expectation of guaranteeing therapeutical efficacy and decreasing severe toxicity of the drug. Several factors were investigated concerning the encapsulation efficiency of TPT liposomes, and pH value of the buffer and cholesterol content were found to have significant effects on the encapsulation and drug retention. This may be due to the structural properties of TPT. TPT contains an N,N-dimethylaminomethyl functional group at the C-9 position (p*K*a = 10.5), an phenolic hydroxyl in the A ring that has a p*K*a of ~6.5–7.0, and a hydrolytically sensitive lactone ring that undergoes a reversible pH-dependent conversion to an inactive carboxylate [33,34]. Therefore, it is present in different forms over a relatively broad pH range, and this complex ionization chemistry of TPT has implications for both drug loading and retention. When the pH value of the buffer is lower, TPT is mainly present in a highly positively charged form, which strongly interacts with EDTA; accordingly, drug loading and retention were satisfactory. However, at higher pH values, TPT is predominantly an anionic phenolic hydroxylate anion in the A ring and a carboxylate that arises from hydrolysis of the D ring, resulting in increased electrostatic repulsion between TPT and EDTA, and thus, unsatisfactory drug retention was observed. Interestingly, when the extraliposomal solution pH of the buffer was in the range of 3.5–7.5, although TPT was present in highly charged zwitterions, the loading efficiency was >88%. This indicates that pH value mainly influenced the interaction between TPT and EDTA intraliposomally rather than through membrane permeation of TPT when the pH of the buffer was <7.5.

It has been reported that in a lipid membrane, incorporation of cholesterol could increase the homogeneity and stability of bilayer; moreover, cholesterol also has an additional benefit of increasing liposome stability in the blood compartment due to reduced lipid exchange with lipoproteins [26,35]. However, a recent report demonstrated that some relatively hydrophobic drugs leaked rapidly from the cholesterol-containing liposomes, due to the cholesterol–drug interactions [27]. In this study, we found that cholesterol is beneficial for TPT loading and retention. The explanation may be that in the present liposomal system, the role of cholesterol in maintaining the stability of bilayer was more predominant than the interaction with TPT.

In addition, to our surprise, in contrast to anthracyclines such as doxorubicin and idarubicin, both the *in vitro* release test and the *in vivo* plasma elimination experiment of TPT demonstrated that compared with $(NH_4)_2SO_4$-L, NH_4EDTA-L showed no superiority in drug retention, indicating that EDTA is only effective in improving the drug retention of anthracyclines rather than camptothecin derivatives. This is probably because TPT does not form a gel-like precipitate with EDTA as doxorubicin or idarubicin does [27]. Even though liposomes prepared by a transmembrane NH_4EDTA gradient did not show superiority in improving TPT retention, they still have irreplaceable advantages regarding storage stability as compared to liposomes formed using a $(NH_4)_2SO_4$ gradient. Our long-term stability experiment showed that the loading ability of NH_4EDTA gradient liposomes stored at 4 °C for 3 months was almost the same as that of liposomes before storage, while $(NH_4)_2SO_4$ gradient liposomes had a decreased loading ability for TPT even at 1 month after storage (Data not shown).

It is worth noting that the toxicity of NH_4EDTA-L was dramatically lower than that of $(NH_4)_2SO_4$-L, regardless of the dosage, and especially the damage to the immune system. This can be partially attributed to the EDTA chelation of heavy metals. It has been reported that heavy metals can inhibit and/or cause deregulation of the immune system, resulting in binding of receptor sites, suppression of proper enzyme functions, reduced antibody response, disordered hormone functions, and undesirable upregulation of the immune system leading to autoimmune disease [23]. Therefore, the damage of antitumor drug-loaded liposomes using a NH_4EDTA gradient on the immune system is reduced probably due to the chelation effect of EDTA. Meanwhile, as we have described earlier, hypercalcinemia is the most common metabolic emergency in tumor patients and always has a serious impact on patient's quality of life. Therefore, when EDTA was used in the formulation, extra calcium may be eliminated due to the chelation effect of EDTA and thereby may reduce this complication and improve patient's quality of life.

So far, we have encapsulated several amphiphilic weak bases using a transmembrane NH_4EDTA gradient. All tested anthracyclines were highly stabilized intraliposomes; while as to other drugs such as TPT, vinorelbine, vincristine, and irinotecan, their retention characteristics are not as satisfied as anthracyclines. However, all of our results showed that liposomes prepared by the transmembrane NH_4EDTA gradient significantly decreased the toxicity of cytotoxic drugs compared with liposomes that were prepared by a $(NH_4)_2SO_4$ gradient, regardless of encapsulation of doxorubicin, epirubicin, vinorelbine, or TPT. These findings lead us to conclude that this particular method will have applications for many anticancer drugs with not only high activity but also high toxicity. It is believed that this encapsulation method will provide a new platform for antitumor drugs previously abandoned because of their severe side effects.

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