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Report

Simple and efficient liposomal encapsulation of topotecan by ammonium sulfate gradient: stability, pharmacokinetic and therapeutic evaluation

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Topotecan (TPT), a topoisomerase I inhibitor, is presently undergoing clinical evaluation worldwide. Previous studies have shown that entrapping TPT within multi-lamellar vesicle liposome can stabilize the lactone moiety, which is structurally important for biological activity. However, low drug:lipid ratios due to the amphipathic character and small entrapment volume in the unilamellar vesicle limits the development of pharmaceutically acceptable liposomal formulation. With an aim to improve on this drawback, we herein describe a method that utilizes the ammonium sulfate gradient to entrap TPT into liposomes. By this method, the encapsulation efficiency was over 90% and a drug:lipid molar ratio as high as 1:5.4 was reached. In comparison with free drug, liposome-encapsulated TPT is more stable in physiological conditions and shows higher *in vitro* cytotoxicity. Because of increased blood circulation time, the initial plasma concentration and area under the plasma concentration of liposomal drugs were 14 and 40 times, respectively, of those of free drug. Furthermore, liposome encapsulation enhanced the antitumor activity of TPT in syngeneic murine C-26 and human HTB-9 xenograft models *in vivo*. At a dose of 5 mg/kg, the tumor growth delay of liposomal formulation was significantly than that of free TPT. Based on these results, we believe that this liposomal TPT formulation is worthy of further clinical study. [© 2002 Lippincott Williams & Wilkins.]

Key words: Ammonium sulfate gradient, drug delivery system, liposome, topotecan.

Introduction

Topotecan (TPT, 9-dimethylaminomethyl-10-hydroxy-camptothecin), a semisynthetic analog of camptothecin (CPT), has enhanced aqueous solubility and reduced protein binding relative to CPT. Its antitumor activity has been demonstrated in preclinical tumor models,[1] and in phase II clinical trials of ovarian cancer,[2] non-small cell lung cancer[3] and colorectal cancer.[4] Similar to CPT and other derivatives, TPT is a cell cycle-specific drug and acts as stabilizer of complex of DNA and topoisomerase I. This results in single-strand break of DNA, which leads to severe DNA damage during subsequent replication followed by cell death.[1,5,6] Therefore, it is advantageous to expose tumor cells to the drug for a prolonged period. This point is supported by clinical observations that patients refractory to TPT exhibited increased response rates when the drug was administered as a low-dose infusion.[7] Unfortunately, TPT is extremely unstable in physiological conditions.[5,8] Conversion of the lactone form into the carboxylate form occurs rapidly at physiological pH.[9] Although the cellular basis of its antitumor activity is still incompletely understood, available data indicates that an intact α-hydroxylactone ring is important both for passive diffusion of the drug into cells as well as for inhibition of topoisomerase I.[5,10,11] Accordingly, efforts have been made to explore factors affecting its structure stability in order to improve the cytotoxic activity.[12–14]

Liposomes have been used for drug delivery in the past three decades. Several studies have shown that liposome encapsulation of anticancer agents can alter tissue disposition,[15,16] reduce blood clearance rate,[17] decrease toxicity and enhance antitumor activity.[18,19] In addition to these advantages, the properties of TPT, i.e. S phase-specific cytotoxicity and fast inactivation at physiological pH, make it worthwhile to develop liposomal TPT. Previous work

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has shown that complex of CPTs with lipid vesicles, composed of DMPC or DMPG, can stabilize its lactone moiety and thereby prevent drug inactivation.[20] However, low drug:lipid ratios due to the amphipathic character and small entrapment volume in the unilamellar vesicle limited the development of pharmaceutically acceptable liposomal formulations.[21,22] To improve trapping efficiency, a strategy that uses a transmembrane ionic gradient to pack drugs within the liposome would be useful. In the present work, therefore, the ammonium sulfate remote-loading method was selected for developing an acceptable liposomal TPT formulation. With this method, higher TPT loading could be achieved. In addition, the low pH environment spontaneously generated after removal of ammonium ions[23] could maintain TPT in its active lactone form. The pharmacokinetic properties and antitumor activity of both free and liposomal TPT were compared in the present work.

Materials and methods

Materials

TPT (SmithKline Beecham, King of Prussia, PA) was obtained as a commercially available product. Distearoyl phosphatidylcholine (DSPC) and cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabaster, AL). The lipids were dissolved in chloroform, sealed in ampoules under argon and stored at −20°C before use. HPLC-grade acetonitrile and glacial acetic acid were purchased from JT Baker (Mallinckrodt Baker, Phillipsburg, NJ). Cell culture materials were obtained from Gibco/BRL (Grand Island, NY). All other chemicals were purchased from Sigma (St Louis, MO).

Cells and cell culture

Cells of HTB-9 (human bladder carcinoma) and C-26 (murine colon carcinoma) were cultured as exponentially growing subconfluent monolayers on 100-mm plates (Corning, Corning, NY) or 75-cm^2 tissue culture flask (TPP, Zollstrasse, Switzerland) in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum and 2 mM glutamine at 37°C/5% CO_2 in a humidified incubator.

Preparation of liposomes

Small unilamellar vesicles (SUV, size less than 100 nm) were prepared by a combination of the standard thin-film hydration method and repeated extrusion in the following way.[24] DSPC:Chol (molar ratio 3:2) were dissolved in chloroform and placed in a round-bottomed flask. The solvent was removed by rotary evaporation under reduced pressure. The resulting dry lipid film was hydrated at 60°C in ammonium sulfate solution [250 mM $(NH_4)_2SO_4$, pH 5.0, 530 mOs] and dispersed by hand shaking at 60°C. The suspension was frozen and thawed 5 times,[25] and followed by repeated extrusion through polycarbonate membrane filters (Costar, Cambridge, MA) of 0.1-μm pore size 3 times and 0.05-μm pore size 7 times by using high-pressure extrusion equipment (Lipex Biomembranes, Vancouver, BC) at 60°C. After extrusion, the extra-liposomal salt was removed by a Sephadex G-50 column (Pharmacia Biotech, Uppsala, Sweden) equilibrated with 100 mM MES buffer solution containing 260 mM NaCl and 2 mM EDTA (pH 5.5, 580 mOs).

TPT encapsulation

TPT was encapsulated into liposome using an ammonium sulfate gradient.[26] After removing the extra-liposomal salt by a Sephadex G-50 column, TPT in powder form was added immediately into the solution at a concentration of 1 mg TPT/10 μmol phospholipid. The mixture of liposome and TPT was incubated in 60°C water bath for 30 min with agitation (100 r.p.m.). After loading, untrapped TPT was removed by Sephadex G-50 gel filtration in 0.9% NaCl solution at pH 6.0 and osmolarity of 286 mOs. For the passive loading method, thin lipid film was hydrated at 60°C in ammonium sulfate solution with 2 mg/ml of TPT. The mixture was then extruded and separated from free drug by Sephadex G-50 gel filtration in 0.9% NaCl solution.

The extent of encapsulation was determined by measuring lipid and TPT concentration. In brief, the final concentration of liposomes was estimated by phosphate assay.[27] The amount of TPT trapped inside the liposomes was determined with a spectrofluorometer (Hitachi F-4500; Hitachi, Tokyo, Japan), after adding 0.8 ml acidic ethanol (0.6 N HCl in ethanol) to 0.2 ml diluted drug-loaded liposomes, using 381 nm as the excitation wavelength and 525 nm as the emission wavelength. Vesicle sizes were measured by dynamic laser scattering with a submicron particle analyzer (model N4+; Coulter, Hialeah, FL). In our preparations, TPT-loaded liposomes contained 90–100 μg TPT/μmol phospholipid.

The particle size of our liposome preparations ranged from 65 to 75 nm in diameter (Table 1).

Release of TPT from liposomes

An aliquot of 100 μl of liposome suspension (0.5 mg/ml). was incubated with 900 μl of PBS (pH 7.4) and human blood plasma (HBP) at 37°C. At time points of 0.083 (5 min), 1, 2, 6, 12, 24 and 48 h, aliquots of 100 μl were withdrawn and the free TPT was removed by Sepharose CL-4B gel filtration. A small aliquot of liposomal TPT was diluted (as necessary) with unbuffered saline. Phospholipid and TPT concentrations were determined by phosphorus analysis and spectrofluorometry (Hitachi F-4500) as described.

Kinetic evaluation of lactone ring opening

The lactone ring opening rates for TPT as a result of hydrolysis were determined by reverse-phase HPLC assays. A drug concentration of 1 mM was prepared in PBS (pH 7.4) at 37°C. At time points of 0.083 (5 min), 0.5, 1, 2, 6, 24, 48 and 72 h, 200 μl of aliquots were withdrawn and mixed with 0.8 ml of methanol. The mixture (20 μl) was injected into HPLC directly for analysis. HPLC analysis of TPT was based on the method described by Warner.[28] The mobile phase consisted of TEAA buffer [2% triethylamine in water (v/v), adjusted to pH 5.5 with glacial acetic acid] and acetonitrile. The ratio of acetonitrile:TEAA buffer (v/v) was adjusted to 12:88. All mobile phases were filtered and degassed using sonication prior to use. For analysis of TPT, the isocratic HPLC system utilized a Shimadzu LC-9A pump, a Waters 717 autosampler and a Shimadzu RF-551 spectrofluorometric detector (excitation wavelength 381 nm, emission wavelength 525 nm). Separation was carried out at ambient temperature using a Waters NovaPak-C18 reverse-phase column (4 μm particle size; 150 × 3.9 mm i.d.). In all instances, a flow rate of 1.0 ml/min was employed.

In vitro antiproliferative effect of liposomal TPT

The antiproliferative effect of TPT-loaded liposomes was determined by MTT assay.[29] Briefly, 5×10^3 tumor cells were seeded into each well of 96-well microtiter plates in appropriate medium. The C-26 and HTB-9 cells were either incubated with free TPT or liposomal TPT for 2 days before the MTT assay. The concentrations of test drugs ranged from 0.01 to 100 μg/ml. As the control, same dose of empty liposome was added into culture medium.

Pharmacokinetics studies in BALB/c mice

Plasma clearance studies were performed with male BALB/c mice (five mice per group). Each mouse was treated with a drug dose of 5 mg/kg in a volume of 10 ml/kg via the lateral tail vein using a 26-gauge needle. Blood samples (0.05 ml) were collected at 0.083 (5 min), 0.5, 1, 2, 6, 12 and 24 h after the i.v. injection. Blood samples were then mixed immediately with 250 μl of 0.5 mM EDTA/PBS followed by centrifuging at 200 *g* for 5 min.The cell pellets were washed once with the same solution and the supernatants were combined. TPT was extracted with 2.5 ml of acidic ethanol (0.6 N HCl in ethanol) and incubated overnight at 4°C to precipitate protein before detection by spectrofluorometer (Hitachi F-4500).

Pharmacokinetic analysis was done by non-linear least-squares analysis using Pkanalyst software (MicroMath, Salt Lake City, UT). The plasma concentration–time data were fitted to a biexponential equation as:

$$C_{(t)} = A_1 e^{-k1t} + A_2 e^{-k2t}$$

where $C_{(t)}$ is the drug concentration (y-axis) at time t (x-axis), k_1 and k_2 are slopes or apparent first-order elimination rate constants, and A_1 and A_2 are the y-intercepts.

The area under the concentration–time curve (AUC) was calculated from the sums of the ratios A_1/k_1 and A_2/k_2. Clearance (CL) was calculated by

Table 1. Characteristics of liposomal TPT preparations

	Drug: lipid (mol: mol)	Encapsulation efficiency (%)	Size (nm)
Active remote loading	1:5.4	93.1 ± 0.8	68.3 ± 16.2
Passive loading	1:146.0	5.23 ± 0.2	73.1 ± 11.8

All data calculated from experiments conducted in triplicate.

dividing dose over AUC. Volume of distribution at steady state (V_{ss}) was calculated using the equation:

$$V_{ss} = \text{Dose} \times \text{AUMC}/(\text{AUC}^2)$$

where AUMC was the area under the product of $C \times t$ plotted against t from time 0 to infinity.

Animal models for therapy study

BALB/c mice and SCID mice (6–8 weeks, weighing 17–20 g) were purchased from the Animal Center at the College of Medicine, National Taiwan University, Taipei, Taiwan. At day 0, BALB/c and SCID mice were injected s.c. with 2×10^5 of C-26 and HTB-9 tumor cells respectively. When the mean tumor diameter reached 5 mm, mice in groups of eight or 10 were randomly divided into four groups. The tumor-bearing mice were treated with free or liposomal TPT at a dose of 5 mg/kg through the tail vein weekly for 2 weeks. For the control groups, mice were treat with equal amounts of empty liposome or normal saline. Tumor size of each mice was measured by caliper and measure calculated by the formula: $a \times b^2/2$, where a=length and b=width (in mm). The study was repeated 3 times and the results were consistent.

Statistical analysis

Statistical comparison between free TPT and liposomal TPT were carried out using Student's t-test. A level of $p < 0.05$ was considered to be indicative of statistical significance.

Results

Improvement on drug encapsulation by active loading

Consistent with previous studies,[21] due to the amphipathic character and small entrapment volume in the unilamellar vesicle, passive loading of TPT by directly hydrating the drug with dried lipid film resulted in low encapsulation efficiency (5%, Table 1). The molar ratio of TPT to phospholipids was merely 1:146. In contrast, when the ammonium sulfate remote loading method was used, the encapsulation efficiency was approximately 90%, which is 18-fold higher than that of the passive loading method. The molar ratio of drug to phospholipids was also increased 27-fold, up to 1:5.4.

Stability of liposomal TPT

Based on Haran's study,[23] the intraliposomal space could maintain at a low pH environment after ammonium sulfate remote loading. Therefore, the active lactone structure would be kept while TPT retain within liposome (Figure 1). For this reason, retention of TPT within liposome was studied. As shown in Figure 2(A), there was no significant change in TPT content of liposomal TPT after 48 h incubation with PBS at 37°C. However, in the presence of 90% HBP, encapsulated TPT leaked out of liposome gradually in a time-dependent manner. It is apparent that the leakage of liposomal TPT is markedly affected by HBP interaction.

Figure 2(B) depicts the changes of lactone percentage in physiological pH as a function of time. Hydrolysis of free TPT proceeded quickly with a short half-life ($t_{1/2}$ value) of about 19.7 min. In contrast, for

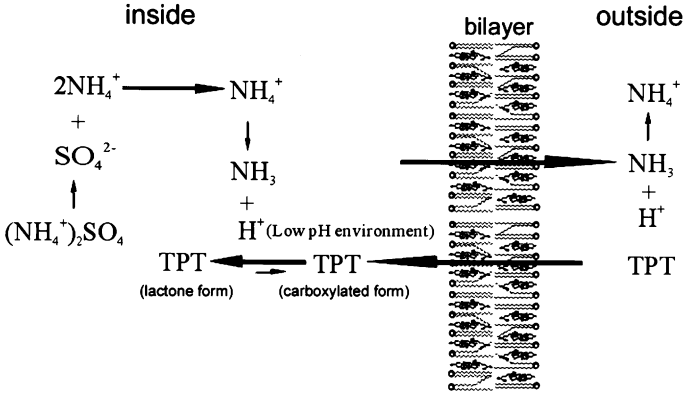

Figure 1. Scheme of liposomal TPT loaded by an ammonium sulfate gradient.

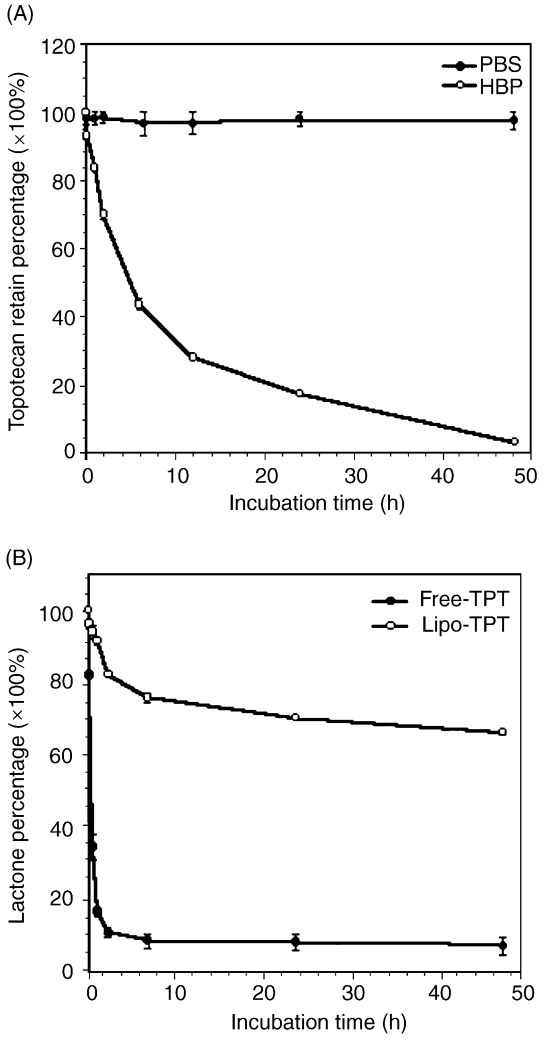

Figure 2. Characterization of liposomal TPT. (A) Release of TPT from liposomes in the presence of PBS or HBP. (B) Kinetic evaluation of the rate of lactone ring opening for free TPT and liposomal TPT in PBS. A drug concentration of 1 μM was incubated in PBS (pH 7.4) at 37°C. At each time point, each sample was manipulated as described in Materials and methods. Each value represents the mean ± SD of three independent experiments.

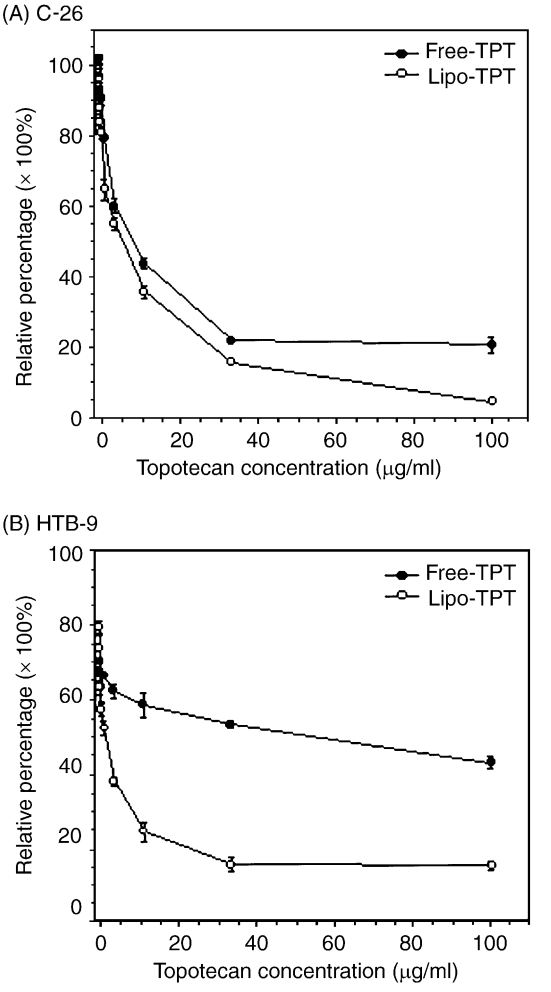

Figure 3. *In vitro* comparison of antiproliferative activities of free TPT and liposomal TPT against (A) C-26 and (B) HTB-9 cells. Cells in 96-well microtiter plates were treated with free TPT and liposomal TPT at doses indicated, and relative cell growth was determined by MTT assay as described Material and methods. Control empty liposomes were dosed according to phospholipid concentration. Each value represents the mean ± SD of five determinations.

liposomal TPT, the stability of the lactone moiety was markedly enhanced by liposome encapsulation. Seventy percent of TPT remained in lactone form after 48 h incubation with PBS (pH 7.4) at 37°C. Clearly, the lactone ring of TPT was notably preserved upon liposome encapsulation.

In vitro toxicity of free and liposomal TPT

Mouse C-26 cells were treated with varying doses of free or liposomal TPT. As shown in Figure 3(A), liposome encapsulation slightly enhance antiproliferation ability of TPT (IC_{50}=4.9±0.7 and 6.7±0.5 μg/ml for liposomal TPT and free TPT, respectively). However, the cytotoxic superiority of liposomal TPT was more pronounce in the HTB-9 tumor cell line (Figure 3B), for which IC_{50}s are 1.6±0.4 and 41.7±6.3 μg/ml for liposomal TPT and free drug, respectively. As a control, empty liposome did not cause any inhibition of cell growth (data not shown).

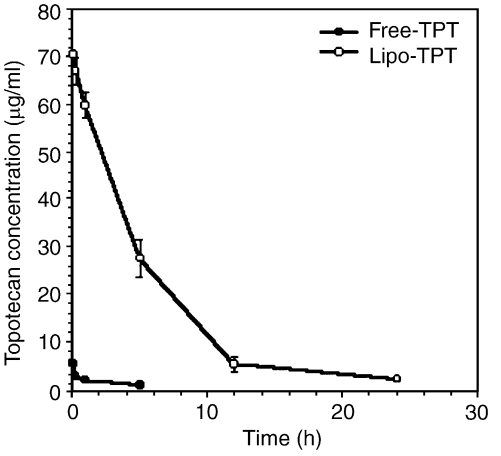

Figure 4. Plasma concentration–time relationship for free TPT and TPT encapsulated in liposomes. Male BALB/c mice were injected i.v. with a 5 mg/kg dose of free TPT or liposomal TPT. Plasma levels of TPT equivalents were determined as described in Materials and methods. Each point represents the mean ± SD of five animals.

Pharmacokinetics of liposomal TPT

The plasma drug concentrations of free or liposome-encapsulated TPT are presented in Figure 4. Following injection of 5 mg/kg free TPT, at 5 min post-injection time, the plasma concentration (C_{max}) of free TPT achieved was 5 μg/ml and it decreased rapidly within 15 min.On the other hand, at 5 min post-dose, the concentration of liposomal TPT achieved was 70.5 μg/ml, which was 14 times higher than that of free TPT. The pharmacokinetic parameters of free and liposomal TPT are compared in Table 2. Liposomal drugs had a 40-fold higher area under the AUC in comparison to free drug. V_{ss} was substantially reduced with liposomes as compared with free drug. CL for liposomes was also decreased 38-fold than free TPT.

Effects of TPT on tumor growth in mice

Because of the improved pharmacokinetic profile of liposomal TPT, we next assessed its antitumor activity with syngeneic C-26 tumor in BALB/c mice and HTB-9 xenograft in SCID mice models.

In. the C-26 tumor model, when mean tumor diameter reached 5 mm, TPT was given at a dose of 5 mg/kg i.v. injection weekly for 2 weeks (Figure 5A). The mean tumor size reached 3925 ± 545 and 3922 ± 529 mm^3 in the saline and empty liposome-treated control respectively at day 32, whereas mice receiving free TPT showed minimal growth delay (mean tumor size = 3094 ± 303 mm^3). In contrast, a significant delay in tumor growth rate was observed

Table 2. Pharmarcokinetic parameters of free and liposomal TPT in BALB/c mice after a single i.v. dose of 5 mg/kg

	Liposomal TPT	Free TPT	Increase
C_0 (mg/l)	70.6	5.0	14 ×
$T_{1/2\alpha}$ (h)	2.1	0.1	
$T_{1/2\beta}$ (h)	2.9	2.6	
AUC (mg · h/ml)	358.4	9.4	40 ×
V_{ss} (l/kg)	0.06	1.88	
CL (l/kg/h)	0.01	0.53	

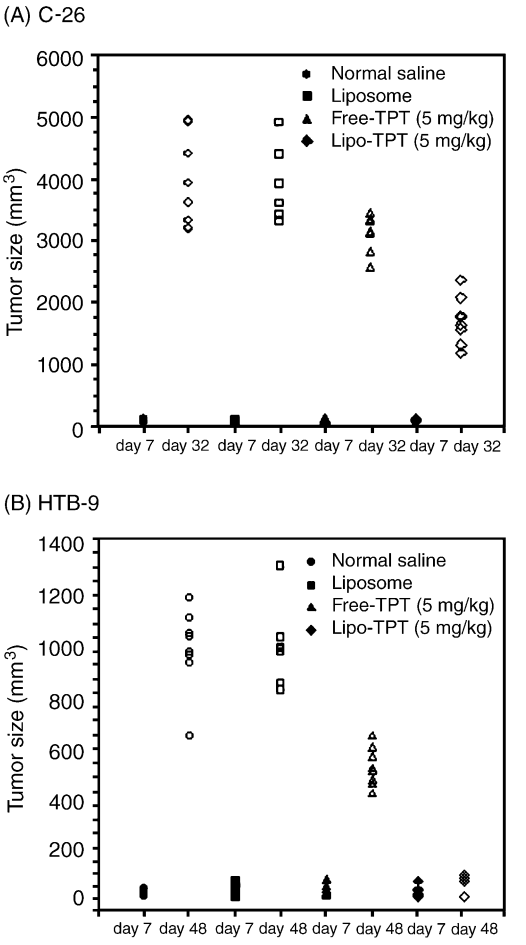

Figure 5. *In vivo* comparison of antitumor activity against (A) mouse C-26 colon carcinoma and (B) human HTB-9 xenograft. Mice were injected s.c. with 2×10^5 tumor cells in one flank on day 0 and randomly divided into four groups when mean tumor diameter reached 5 mm.Each group of mice was given a tail vein injection of different formulations at 5 mg/kg. Control groups received the same volume of normal saline and empty liposome. Tumor size was measured as described in Materials and methods.

in the liposomal TPT group (mean tumor size=$1668 \pm 386\,mm^3$). This therapeutically superiority of liposomal TPT was also observed in HTB-9 tumor xenografts (Figure 5B). At day 48, the mean tumor sizes for saline and empty liposome were 969 ± 152 and $933 \pm 214\,mm^3$, respectively. The mean tumor size was slightly affected by free TPT treatment (mean tumor size=$561 \pm 171\,mm^3$). However, liposomal TPT caused tumor remission among half of mice treated. Thus, liposomal TPT exhibited better therapeutic efficacy versus free TPT and the control groups in the animal tumor models.

Discussion

Complexes of CPT with lipid can stabilize the lactone moiety and prevent its inactivation.[20] Correspondingly, entrapment of TPT within the liposome can prolong the half-life of the drug.[21] However, the obstacle to the development of pharmaceutically acceptable liposome formulations is low CPT:phospholipid ratios, especially the TPT.[20] Although Tardi and his colleagues had used an ionophore-generated proton gradient to enhance the encapsulation of TPT into liposome,[30] the drug-loading procedure is slightly complicated. In this study, we adopted ammonium sulfate remote loading to achieve higher entrapment levels. This technique was applied for loading of amphipathic weak bases such as doxorubicin for many years. It takes advantage of the large difference of ammonium sulfate gradient, $[(NH_4)_2SO_4]_{lip} > [(NH_4)_2SO_4]_{med}$, across the lipid bilayer to generate a high driving force to active loading drugs into the aqueous compartment of liposome efficiently.[23] Therefore, there is no need for buffer or pH titration during the procedure and drug encapsulation is easier. This was illustrated by the 18-fold encapsulation efficiency and a drug:lipid ratio in excess of 27-fold greater than that achieved by the direct hydration method.

In addition to the increased trapping efficiency, another important advantage provided by ammonium sulfate loading is the acidic intraliposomal environment formed spontaneously during drug encapsulation. Because CPT undergoes a pH-dependent reversible hydrolysis from active lactone form (at low pH) to inactive carboxylate form (at high pH),[9,12] the drug molecules should be kept in a low pH environment so that their biological activity can be preserved. According to the study of Haran previously, the pH of internal liposomal aqueous space will drop below 5 after complete removal of external ammonium sulfate as indicated by pH-sensitive fluorescence dye.[23] It implies that the intraliposomal environment created by ammonium sulfate loading is suitable for maintaining the lactone form (Figure 1). As showed in the TPT hydrolysis test, free TPT displayed a rapid hydrolysis kinetic with a short half-life of about 20 min and had 8.7% of the lactone form at equilibrium, whereas liposome-encapsulated TPT exhibited an enhanced stability with about 70% of lactone form at equilibrium (Figure 2B). It should be noted that up to 30% of liposome-encapsulated TPT exists in the carboxylate form when incubated in PBS, although there was no significant TPT loss in leakage tests (Figure 2A). Theoretically, because of the acidic environment, a higher proportion of TPT should exist in the lactone form if all the drug molecules have been entrapped inside the liposome. This suggested that a portion of TPT, although still associated with phospholipid, might contact with the external basic buffer and be subjected to hydrolysis.

Although the enzyme level may affect cellular sensitivity to topoisomerase I inhibitors,[31,32] the effectiveness of CPT treatment also depends on the form of cells exposed. For the reason that the intact lactone moiety is structurally important for biological activity,[5,11] liposomal TPT is much more effective than free drug to inhibit C-26 and HTB-9 cells growth (Figure 3). The enhanced antitumor activity of liposomal TPT can be accounted by its ability to maintain a higher portion of active lactone form, whereas free TPT is quickly hydrolyzed into its inactive carboxylate form.

Pharmacokinetic analysis indicated that liposomal-TPT had a smaller volume of distribution, which is close to total blood volume, but there was little difference in $t_{1/2\beta}$. This could be explained by the poor retention of TPT in the presence of serum. Although some reports argued that liposomes comprised of saturated neutral phospholipids and a high percentage of cholesterol can reduce protein interaction and drug leakage;[33–35] nevertheless, TPT escaped from the DSPC/Chol-based liposome used in this study faster than other drugs, such as vincristine[36] or doxorubicin.[37] We do not have a good explanation for this phenomenon. One possibility is that TPT does not form a gel-like precipitate with ammonium sulfate as doxorubicin does.[38]

Preclinical *in vitro* and *in vivo* studies indicated that prolonged exposure to low-dose topoisomerase I inhibitors is the most efficacious.[39–41] As the result of pharmacokinetic tests, the overall plasma AUC of liposomal TPT was around 35 times that of free drug. This altered distribution may reduce the toxicity to

normal tissues and the higher plasma AUC may facilitate drug distribution to tumor. The improvement in pharmacokinetic parameters was reflected in the therapeutic efficacy (Figure 5). In the C-26 model, the tumor size decreased from $3094 \pm 303\,mm^3$ of free TPT-treated mice to $1668 \pm 386\,mm^3$ of liposomal TPT treated. More significantly, in the HTB-9 xenograft model, the total tumor regressions were observed in half of liposomal TPT-treated mice. It might be argued the similar results would be achieved with a higher dose or several dose regimens of free drugs. However, in the initial animal study, the dose-dependent antitumor effects and various dose regimens of free and encapsulated TPT were been examined on C-26 tumor-implanted mice (data not show). According to the results in such experiments, we found that for 2 and 5 mg/kg free drug treatment the final tumor volumes were 1828 ± 462 and $1857 \pm 300\,mm^3$, respectively, at the end of the experiment. There are no significant difference between these two groups and the control group (final tumor volume $2009 \pm 257\,mm^3$). In contrast, after treatment with 2 and 5 mg/kg liposomal TPT, the tumor growth was markedly delayed and the final tumor volumes were 1298 ± 211 and $1001 \pm 210\,mm^3$, respectively. Even more, the therapeutic effects of liposomal TPT administrated once weekly for 2 weeks were superior to those mice administrated free TPT twice weekly for 2 weeks (final tumor volumes $1709 \pm 287\,mm^3$ for 2 mg/kg and $1529 \pm 387\,mm^3$ for 5 mg/kg). In addition, results of TPT hydrolysis and *in vitro* cytotoxicity tests suggested that the increased therapeutic activity achieved with liposomal TPT is not only due to a change of the pharmacokinetics, but also due to enhanced drug stability. Therefore, the improvement of therapeutic efficacy may not be achievable with multi-dose regimens or continuous i.v. infusion of free drug.

In summary, the use of active ammonium sulfate remote loading to encapsulate TPT has several advantages. The powerful ionic gradient driving force considerably increased encapsulation efficiency. Furthermore, acidification of liposomal internal space during the loading process tips the equilibrium to the biologically active lactone form. The low activity of free TPT against C-26 and HTB-9 tumors can be markedly enhanced after encapsulation in liposome. Although the retention of TPT within the DSPC/Chol-based liposome was shorter than that observed for doxorubicin, the improvement of pharmacological properties still significantly contributes to therapeutic efficacy. Further studies on the behavior of liposomal TPT in circulation will be important to design formulations with better therapeutic activity.

Conclusion

The use of ammonium sulfate ionic gradient loading provides a simple and efficient method to formulate liposomal TPT for pharmaceutical use. In addition, the stability and antitumor activity of TPT can be markedly enhanced after liposome encapsulation.

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