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Development and characteristics of temperature-sensitive liposomes for vinorelbine bitartrate

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ABSTRACT

A novel liposome with temperature-sensitivity for vinorelbine bitartrate (VB) was designed to enhance VB targeted delivery and antitumor effect. Liposomes without drugs were prepared by thin film hydration, and then VB was entrapped into liposomes by pH gradient loading method. The mean particle size of the liposomes was about 100 nm, and the drug entrapment efficiency was more than 90%. Stability data indicated that the liposome was physically and chemically stable for at least 6 months at 4 °C. In vitro drug release study showed that drugs hardly released at 37 °C; while at 42 °C, drugs released quickly. For in vivo experiments, the lung tumor model was established by subcutaneous inoculation of cell suspension on mice, liposomes and free VB were injected i.v. in mice, followed by exposure the tumors to hyperthermia (HT) for 30 min after administration. The ratio of inhibition tumor of temperature-sensitive liposomes group was significantly higher than the normal injection group. Combining temperature-sensitive liposomes with HT enhanced the delivery of VB and, consequently, its antitumor effects. This liposome could potentially produce viable clinical strategies for improved targeting and delivery of VB for treatment of cancer.

1. Introduction

Vinorelbine bitartrate (VB) is a semi-synthetic drug for chemotherapy that has a wide anti-tumor spectrum, and is especially effective in advanced breast cancer (ABC) and advanced/metastatic nonsmall cell lung cancer (NSCLC) (Wan et al., 2008). An injection of vinorelbine (Navelbine® IV) developed by Pierre Fabre Medicament France is now widely marketed to treat NSCLC and ABC around the world. However, Navelbine® IV is not an optimal form of formulation for vinorelbine, because vinorelbine has a vesicant action to cause venous irritation and phlebitis when directly administered as an aqueous solution (Yoh et al., 2004). The symptoms of erythema, pain at the injection site, vein discoloration and tenderness along the vein are often observed clinically (Mare et al., 2003). Thus, a new strategy is needed to reduce the venous irritation produced by vinorelbine injection (Zhang et al., 2008).

In recent years, much attention has been focused on drug delivery systems (DDS) for cancer chemotherapy. Liposomes have been studied extensively as a DDS, Yatvin et al. and Weinstein et al. (Ono et al., 2002) reported application of liposomes which allows for the control of drug release from liposomes according to the physico-chemical nature of the phospholipid membrane. The features of liposomal drug, which include reduced toxicity and/or increased efficacy due to enhanced accumulation at disease sites, has resulted in the development of a number of antifungal and anticancer liposomal drug formulations (e.g. Ambisomei, ABELCETi, Doxili, DaunoXomei) that have received regulatory approval and have demonstrated significant clinical utility (Kong et al., 2000a,b).

However, specific targeting and controlled release of drugs using liposomes is still a major challenge because current liposomal formulations have been demonstrated to reduced toxicity but do not increase the therapeutic efficacy significantly (Cagnoni, 2002; Judson et al., 2001). Temperature-sensitive liposomes (TSL) were developed for specific site targeting (De Smet et al., 2010). TSLs are able to release encapsulated molecules near their phase transition temperature (T_m), where the lipid membrane shows a transition from a gel to a liquid crystalline phase (Lindner et al., 2004; De Smet et al., 2010). Since the initial description of thermal control of drug delivery systems using TSL by Yatvin et al. (1978), the use of TSL as a drug delivery system has been an area of increasing interest in pharmaceutics (Weinstein et al., 1998; Gaber et al., 1996; Kong et al., 2001). It has been shown that drug encapsulated in thermosensitive liposomes accumulates fourfold more efficiently in tumors heated at 42 °C than in unheated control tumors (Gaber et al., 1996). TSLs also have an enhanced permeation and retention

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effect for tumor vasculature combined with hyperthermia treatment (HT) (Allen et al., 1991), thus, the combination of TSL and HT can increase effect of drugs loaded.

HT has been tried clinically to treat solid tumors, in combination with drug delivery systems, because it can synergistically increase tumor cytotoxicity in combination with chemotherapy and radiotherapy (Ishida et al., 2000; Meyer et al., 2001; Hee et al., 2006). HT generally supports TSL via two routes. First, HT facilitates extravasation of TSL into the heated tumor tissue; second, HT induces controlled drug release of TSL in the heated region (Kong et al., 2000a,b, 2001). For clinical application, where intratumoral temperature does not exceed 42 °C during the treatment time, the need for TSLs for mild HT in the range of 39–42 °C is obvious (Wendtner et al., 2002; Hildebrandt et al., 2002; Anscher et al., 1997).

Herein, in present work, a novel thermosensitive liposome was evaluated in vitro and in vivo for delivery of VB at HT. In vitro drug release had thermosensitive characteristics, changed significantly with alteration of medium temperature. In vivo, combining temperature-sensitive liposomes with HT enhanced the delivery of VB to specific site and antitumor effects. This liposome could potentially produce viable clinical strategies for improved targeting and delivery of VB.

2. Materials and methods

2.1. Materials

Dipalmitoyl phosphatidylcholine (DPPC), monopalmitoyl phosphatidylcholine (MPPC), 1,2-distearyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethyleneglycol)-2000] (DSPE-PEG2000), Egg yolk phosphatidylcholine (EPC), and cholesterol (Chol), were purchased from Avanti Polar Lipids and were used without further purification. Vinorelbine bitartrate (VB) was purchased from Min Sheng pharmaceutical limited company, Hang Zhou, China. All other reagents were analytical grade.

2.2. Preparation of liposomes

Two different liposomal formulations were prepared, temperature-sensitive liposomes (TSL) composed of DPPC: MPPC: DSPE-PEG2000 = 86:5:4(molar ratio) and non-temperature-sensitive liposomes (NTSL) composed of EPC: Chol: DSPE-PEG2000 = 75:50:5 (molar ratio), according to previous report with some modifications (De Smet et al., 2010). Briefly, the phospholipids were dissolved in chloroform, and the solvent was evaporated under vacuum using a rotator RE-2000 (Ya Rong Biochemical Instrument Factory, China) to form a thin film on the inner walls of the round-bottomed flask. The film was dried to remove residual organic solvent and vacuum dried overnight. Hydration of the lipid film was carried out using citric acid–sodium citrate buffer (300 mM, pH 4) at 58–60 °C. During the hydration procedure, rotator was used (150 rpm) to revolve the round-bottomed flask and facilitate suspension of the lipid film into the hydrating medium. The multilamellar vesicles were subjected to extrusion through polycarbonate membrane (Whatman) using an extruder (EmulsiFlex-C3, Avestin, Canada), to obtain liposomes with a uniform size.

Liposomes obtained by above method was eluted through dextran gel (G50) column, using phosphate buffer saline (200 mM, pH 6.4) as eluent. As a result, liposomes with pH gradient between interior and extrinsic phase were developed.

Liposomes containing VB were prepared by pH gradient method. VB solution was directly added into liposomes suspension, and incubated for about 30 min.

2.3. Quantification of VB

HPLC methods were used for the analysis of VB. Drug content analysis was performed using Akasil C18 (4.6 mm × 250 mm, 5 μm, Agela Technologies) HPLC column at 40 °C. The mobile phase was a mixture of methanol solution containing 0.2% decane sulfonic sodium and 0.05 M natrium biphosphoricum water solution (80/20, v/v), at a flow rate of 1 ml/min. Sample injection volumes were 20 μl and VB detection was performed using UV detector (Waters2487, USA) at a wavelength of 267 nm.

2.4. In vitro characteristics of liposomes

2.4.1. Determination of encapsulation efficiency (EE)

Entrapment efficiency of VB in liposomes was determined by a modified minicolumn centrifugation method using dextran gel (Sephadex G-25) column (Zhang et al., 2005). First, dextran gel was allowed to swell in distilled water for 24 h, and the swelled gel was added into column (plastic injector), then the column was centrifuged for 2 min at 350 × *g* using a tabletop centrifuge to remove water. The liposome sample 0.2 ml was introduced into the dry column and centrifuged at 350 × *g* for 2 min to separate free VB from the liposome entrapped drug; 0.2 ml distilled water was introduce into column and centrifuged at 350 × *g* for 2 min to elute residual liposomes, repeated above operation twice. And then, the eluted liposomes were collected and analyzed by HPLC for VB concentration as described previously. What's more, liposome recovery was achieved almost 100% by optimization of minicolumn methods during sample preparation.

The entrapment efficiency was determined by comparing the VB concentration of the eluted sample with that of liposomes sample prior to column chromatography.

The following equation was used to calculate the encapsulation efficiency:

$$\text{Encapsulation efficiency} = \frac{\text{VB}_{\text{aftercolumn}}}{\text{VB}_{\text{beforecouumn}}} \times 100\%$$

2.4.2. Photon correlation spectroscopy

The diameter of the liposomes was determined using photo correlation spectroscopy (Nanophox, Sympatec GmbH, Germany). Liposomes suspension was diluted with distilled water; laser intensity was adjusted to 50–60%; desired temperature was 25 °C; measuring mode was cross correlation.

2.4.3. Morphology of liposomes

The morphology of the liposome was observed by transmission electron microscopy (TEM). Liposomes were diluted with distilled water and dropped on a copper grid and air-dried for 10 min at room temperature after removing the excessive sample with filter paper. After adhesion of liposomes, 2% phosphotungstic acid was dropped onto the grid as a staining solution. The excess staining solution was removed with filter paper. Then, the sample was air-dried at room temperature, and then observed by TEM (JEM-1010, JEOL Ltd., Tokyo, Japan).

2.4.4. Differential scanning calorimetry (DSC)

The phase transition temperatures of the lipid membranes were determined with differential scanning calorimetry (Q200 differential scanning calorimeter, TA Instruments, USA) during heating with 2 °C/min from 30 °C to 50 °C.

2.5. Release of VB from liposomes, in vitro

In vitro, drug release study was conducted at 37 °C and 42 °C, respectively to examine thermo-sensitivity of liposomal VB. Briefly;

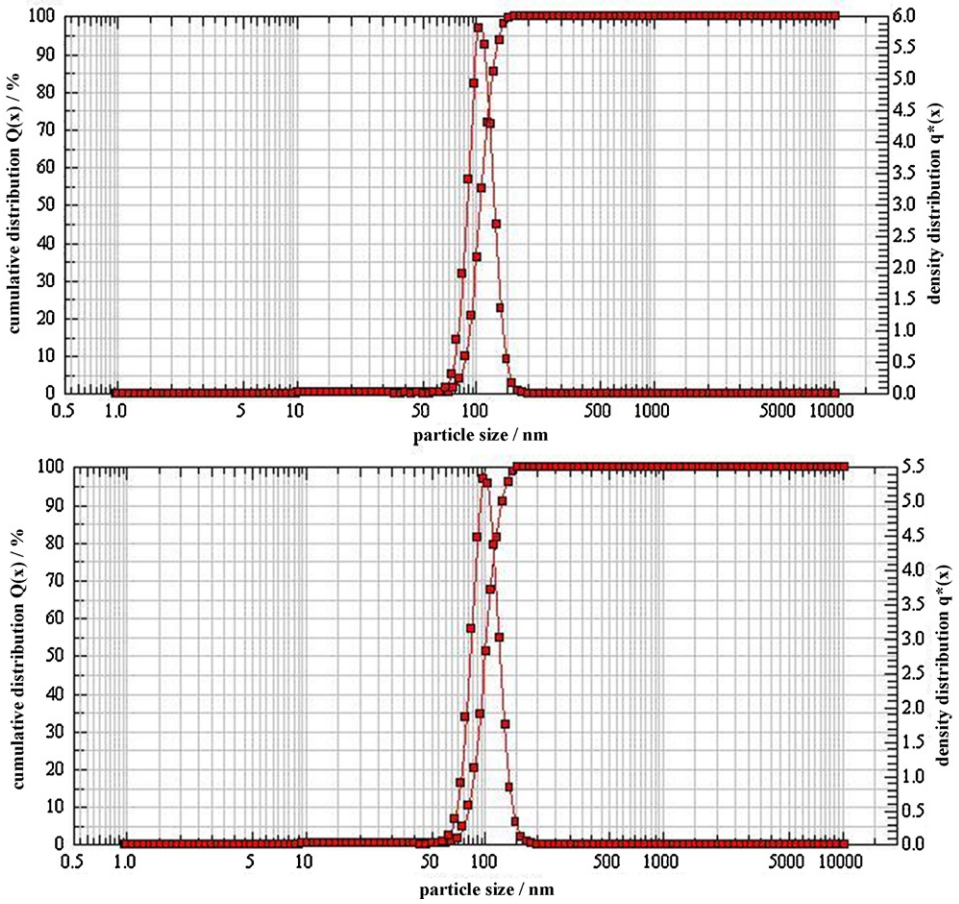

Fig. 1. Particle size distribution of liposomes (A: TSL without VB; B: NTSL containing VB).

liposomal VB sample 2 ml was placed into 20 ml distilled water, stirred and heated at 37 °C and 42 °C, respectively. At various time intervals, aliquot samples were withdrawn and separated by mini-column centrifugation, liposomes containing residuary drug were obtained and its drug concentration was determined out by HPLC method.

2.6. Short-term and long-term stability studies of TSL

The short-term stability study was conducted to evaluate the liquid form of TSL at room temperature for up to 5 d. The long-term stability was conducted to monitor physical and chemical stabilities of the liquid form of TSL at 2–8 °C. The stability parameters, such as, pH, drug concentration, particle size distribution and entrapment efficiency were determined as a function of the storage time.

2.7. Pharmacodynamics of liposomes

2.7.1. Establishment of tumor model

Tumor model on mice (Kunming mouse, purchased from center of laboratory animal, Academy of military medical sciences, China) was established. Inoculation of tumor was carried out by cell suspension method. Lewis malignant cell in exponential phase of growth was digested, counted number, centrifuged and cell suspension (1×10^7 ml^{-1}) was developed with quantitative PBS, placed on ice for the next step. Every mouse was inoculated with 0.2 ml cell suspension by right axilla subcutaneously injection.

2.7.2. Experimental groups

Tumors were allowed to grow about 10 days when their average volume was about 0.15 cm^3 before experiment. According to volume of tumor, 40 mice bearing tumor were randomly divided into four groups. Control group (CG) received tail i.v. of physiologic saline; TSL group was administrated tail i.v. of TSL suspension; NTSL group received i.v. of NTSL suspension; common injection group (IG) was given i.v. of injection of VB (Hao sen pharmaceutical company, Jiang Su, China). After then, tumor location of the four groups was subjected to HT for 30 min, at 42 °C.

2.7.3. Experimental procedure

The complete treatment was as follow. Animals were anesthetized with sodium pentobarbital (50 mg/kg) by intraperitoneal injection. According to experimental groups, different formulations of drugs were administrated by tail vein injection: dosage was 10 mg/kg. After injection, the animal was placed immediately on heat insulation board with holes and tumor was above the holes, which was temperature-controlled. Temperature of holes was controlled at about 42 °C to give HT to tumor location for 30 min. Animals were subjected to treatment every 72 h, three treatment was given in all. During treatment, major and minor diameter of tumor was determined every day, volume was calculated according to formula 1, where *Ma* was major diameter, *Mi* was minor diameter. Three days after final treatment, mice were executed. Their tumor was deprived and weighed. According data determined, growth curves of tumor were drawn and tumor inhibition rate (IR) was calculated as formula 2, where *m* was average weight

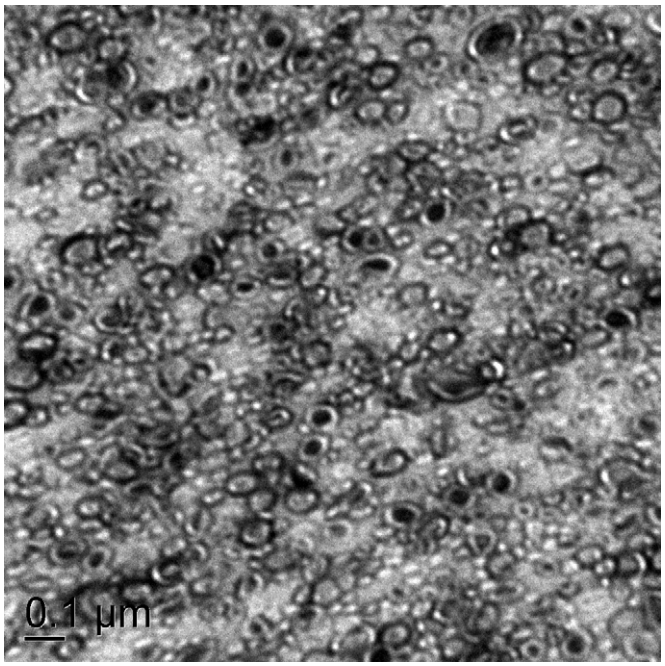

Fig. 2. TEM image of TSL.

of tumor in control group, m_t was average weight of tumor in other experimental groups.

$$V = \frac{1}{2} \times Ma \times Mi^2$$

Formula 1

$$IR(\%) = \left(1 - \frac{m_t}{m}\right) \times 100\%$$

Formula 2

2.7.4. Histological observation

The deprived tumor was fixed in formalin and sliced by paraffin imbedding. The slice was stained with hematoxylin and eosin (H&E) for histopathological analysis.

2.8. Statistical analysis

Statistical analysis was performed by Student's *t*-test for two groups and one-way ANOVA for multiple groups. All results were expressed as the mean ± standard deviation (SD). Probability (p) of less than 0.05 is considered statistically significant.

3. Results

3.1. Characteristics of liposomes

The two liposomal formulations containing VB were prepared by using the same protocol by the active pH gradient method. The liposomes showed a hydrodynamic radius of around 100 nm as evidenced from photon correlation spectroscopy and was semi-transparent with light-blue opalescence. The concentration of VB was about 2.0 mg/ml; EE of the liposomes was more than 90. Compared with liposomes without VB, liposomes entrapped VB have no changes in appearance and particle diameter as shown in Fig. 1. The negative stain transmission electron microscopy images of TSL are presented in Fig. 2. The image showed that the liposomes were spherical, which ranged in size from 80 to 120 nm. The particle size was comparable to the results obtained by PCS technique.

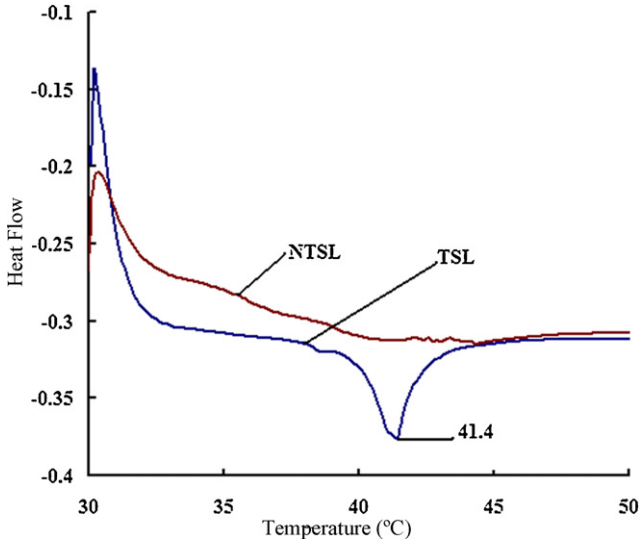

Fig. 3. The DSC of TSL and NTSL.

DSC data (Fig. 3) showed that the phase transition temperature (T_m) of TSL is about 41.4 °C, while NTSL had no obvious phase transition within 30–50 °C.

3.2. VB release from liposomes in vitro

In order to determine rate of drug release changes with temperature alter, drug release was determined at 37 °C, below T_m of TSL and 42 °C, above T_m, respectively. At 37 °C, there was hardly drug to release from TSL, drug hardly released from NTSL within 30 min (Fig. 4.). Meanwhile, when temperature was 42 °C, more than 60% drug released from TSL, however, less than 10% released from NTSL. Therefore, for TSL when temperature was just below the beginning of the T_m as found by the Fig. 3, no VB was released; when the T_m was achieved, most of drug was released. Drug release of TSL changed significantly at T–T_m increased. Thus, in vitro drug release of liposomes certified thermo-sensitivity of TSL, which was coincident with result of DSC for TSL.

3.3. Stability

The short-term stability study showed that the TSL was physically and chemically stable for up to 4 days at room temperature

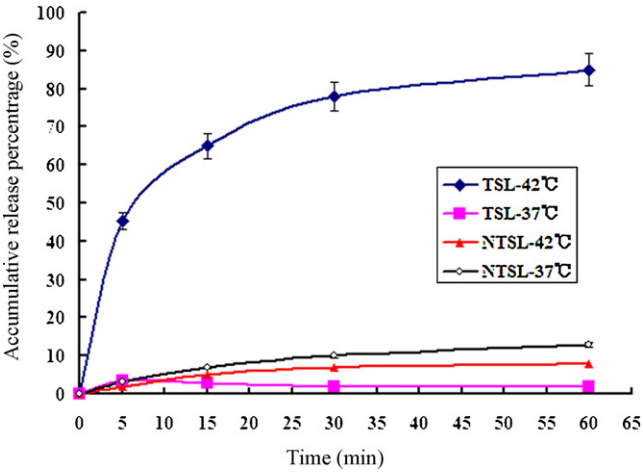

Fig. 4. Drug release of liposomes over time.

Table 1
Stability data of TSL stored at room temperature.

Time (day)	pH	Drug concentration (%)	Particle size (nm)	EE (%)
Undiluted				
0	7.58	100.9	102	96.3
1	7.58	99.6	102	97.2
2	7.56	100.3	100	96.4
3	7.58	100.8	101	95.5
4	7.55	101.0	103	96.4
Diluted 5-fold with saline				
0	7.38	101.9	100	95.7
1	7.28	98.9	101	96.8
2	7.36	100.2	99	97.8
3	7.23	99.1	100	95.9
4	7.25	100.6	98	98.3

All values are mean values of duplicate samples for each time point. Drug results are percent of the initial concentrations as measured at time zero.

(Table 1). There was no significant change in liposome size, pH, drug entrapment efficiency, and drug contents. Since VB is usually given to the patient as intravenous infusion, further dilution of the product is necessary to adjust the dose and drug infusion rate at a given time. The result of the dilution study also demonstrated that 5-fold diluted TSL product stored at room temperature was physically and chemically stable for up to 4 days, respectively (Table 1). The mean liposome vesicle diameter remained unchanged within this time. The entrapment efficiency remained the same regardless of the storage time and conditions. There was no appreciable change in pH for the diluted samples over 4 days study period at room temperature (Table 1). It was concluded that the TSL product can be further diluted up to 5-fold and used within 72 h at clinical setting.

The long-term stability showed that TSL was physically and chemically stable at 2–8 °C up to 6 months (Table 2). The drug contents were within the specifications (≧95% of initial) set for the product for up to 6 months at storage conditions. No significant changes in mean particle size, pH and drug entrapment were observed during the course of stability study.

3.4. Pharmacodynamics

The goal of this study was to compare tumor growth (10 days after first treatment) or inhibition rate (IR) across drug formulations, when all groups received HT at 42 °C. As Fig. 5 shows, all drug formulations in combination with HT were statistically more effective than control group (HT alone, 1335 mm^3, $p < 0.05$); the tumor volume of NTSL (560 mm^3) and IG (660 mm^3) were not statistically different ($p = 0.061$); the tumor volume of TSL group (285 mm^3) was statistically smallest. Thus, TSL group showed the most growth delay. Furthermore, inhibition rate (IR) was used to compare effect across formulations, Table 3 indicate the results. IR of IG group was lowest (53.1%), NTSL group was higher (61.8%), but there was no statistical difference from IG group ($P > 0.05$), and TSL group was highest (77.6%, $P < 0.05$).

Table 2
Stability data of TSL stored at 2–8 °C.

Time (month)	pH	Drug concentration (%)	Particle size (nm)	EE (%)
0	7.58	98.9	102	96.2
1	7.58	101.2	102	97.3
2	7.56	100.8/	100	96.1
3	7.58	100.1	101	95.4
4	7.55	99.9	103	96.8
5	7.53	98.2	102	95.9
6	7.54	99.6	100	98.1

All values are mean values of duplicate samples for each time point. Drug results are percent of the initial concentrations as measured at time zero.

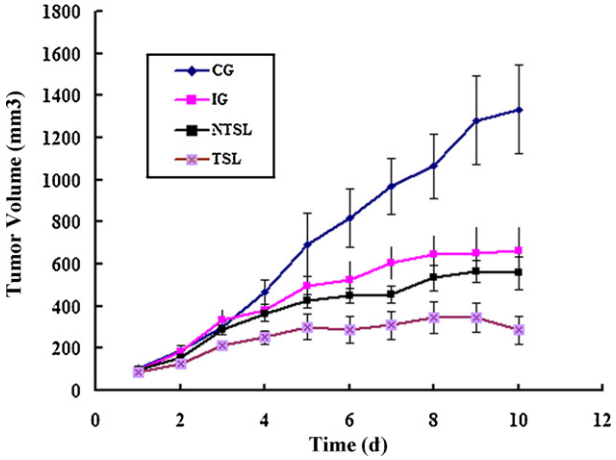

Fig. 5. Tumor volume growth curves.

Histological transmutation (Fig. 6) of deprived tumor was observed after stained with H&E. In CG, the appearance of tumor cell was integral and volume was bigger, intensive, much different volume nucleus were watched, chromatin was coarse and anachromasis (a). Most of tumor cell in TSL group necrosed, nuclear fragmentation and dissolution, disappearance of cellular structures, homogeneous acidophilia alteration could be observed (b). In NTSL group, pycnosis and slight necrosis of part tumor cell nucleus were observed (c). A small quantity of tumor cells in free drug group fragmented, chromatin was anachromasis relatively (d).

4. Discussion

Two liposomal formulations: denoted TSL (temperature-sensitive liposomes) and NTSL (non-temperature-sensitive liposomes) for comparison were prepared using identical procedures. The difference between the two liposomal formulations is the phospholipid composition of the liposomal membranes. The main composition of TSL is DPPC, whose phase transition temperature (T_m) is about 41.9 °C (Mills and Needham, 2005), in the range of mild HT. MPPC, lysolipid, which can accelerate drug release and DSPE-PEG2000, which can prolong retention in vivo of liposomes are the rest composition. On the contrary, the main composition of NTSL is EPC and its T_m is subzero, not in the range of mild HT.

Transmembrane gradient-loading methods (Drummond et al., 2009) have been used to actively encapsulate amphipathic weak bases into liposomes at relatively high efficiencies. These include simple pH gradients (Mayer et al., 1985; Webb et al., 1995), ammonium gradients (Haran et al., 1993), and $MnSO_4$ gradients (Cheung et al., 1998). VB is amphipathic weak bases, so it could be loaded into liposomes by pH-gradient. The pH gradient had been developed in the process of preparing liposomes by eluting dextran gel (G50) column, phosphate buffer saline used. This method was simple, quick and obtained high EE, more than 90%.

According to previous report (Kong et al., 2000a,b), thermosensitive liposomes were divided into two classes: traditional

Table 3
Volume, mass and inhibition rate of tumor ($n = 10$).

Group	Volume (mm^3, $x \pm SD$)	Mass (g, $x \pm SD$)	Inhibition rate	P^1	P^2
CG	1335.2 ± 211.8	1.04 ± 0.68			
TSL	285.9 ± 64.9	0.23 ± 0.17	77.6%	0.025	0.043
IG	661.6 ± 115.8	0.48 ± 0.26	53.1%	0.031	
NTSL	558.9 ± 78.4	0.39 ± 0.16	61.8%	0.03	0.061

The P^1 was the results compared with CG; The P^2 was the results compared with IG.

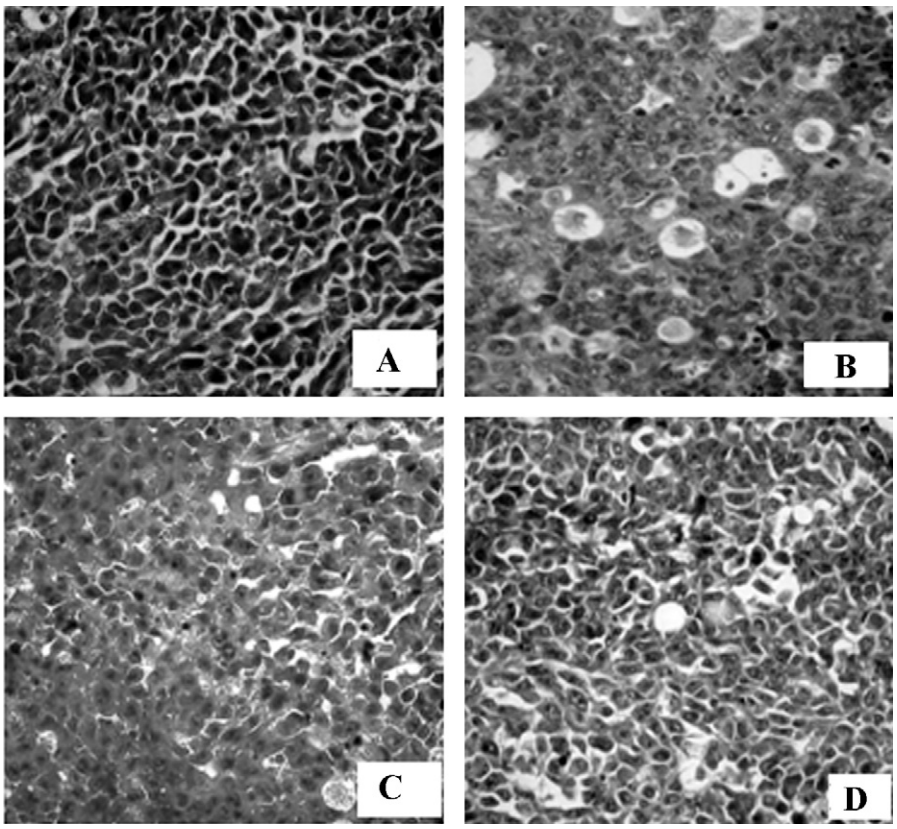

Fig. 6. Histological transmutation of tumor after stained with H&E (A: CG; B: TSL; C: IG; D: NTSL).

thermosensitive liposomes (TTSL) triggers in the range of 42–45 °C and bear lower rate of drug release; low temperature sensitive liposome (LTSL) triggers in the range of 39–42 °C and most drugs are released within relative short time. Furthermore, the TTSL starts drug release in the range of 43–45 °C (Needham et al., 2000), which is not clinically optimized because most clinical HT treatments yield nonuniform heating at 40–41 °C and ranges from 40 to 43°C (Kong et al., 2000a,b). As discussed above, the LTSL is a novel liposome composition that incorporates lysolecithin into the gel-phase lipid membrane, which acts to slightly lower the phase transition temperature of the lipid mixture to 39–42 °C (Anyarambhatla and Needham, 1999). At this lowered transition temperature the drug starts to be released into a temperature range that is clinically attainable in the tumor (Needham et al., 2000). Herein, the TSL for VB is exactly LTSL, whose T_m is 41.39 °C (Fig. 3) and released most of drugs within 30 min when temperature was 42 °C, however, under physiological temperature TSL for VB were stable, there was hardly dug released. This result illustrated that the drug release rates of liposomal drug formulations allowed for stability in the circulation, but release the drug upon reaching the heat location. On the contrary, under both physiological temperature and 42 °C, NTSL had no drug released.

A number of antifungal and anticancer liposomal drug formulations have received regulatory approval. However, the extension of liposome technology to other drugs is often complicated by rapid release of the drug from the liposome following loading, such as the antibiotic ciprofloxacin tend to leak out rapidly (Lasic et al., 1995; Maurer et al., 1998). The efficacy of liposomal formulations of certain drugs, such as anticancer agents, can be extremely sensitive to drug leaking, which means the slowest leaking systems exhibit the slowest leaking systems exhibiting the best efficacy profiles (Boman et al., 1998; Charrois and Allen, 2004). The design of truly optimized liposomal drug delivery systems therefore requires drug retention for long. In this study, TSL was high stable, more than 95% drug were still retained in liposomes for up to 6 months. No significant changes in mean particle size, pH and drug content during the course of stability study.

HT treatment has been tried clinically to treat solid tumors, in combination with liposomes. Kong et al. (2000a,b) reported that it was possible to delineate the mechanism between HT and liposomal drug: HT cytotoxicity, HT-drug interaction, HT-induced liposomal delivery, and HT-triggered liposomal drug release in achieving antitumor activity. This study was designed to compare therapy effect of different formulations for VB. Thus, to eliminate the effect of HT cytotoxicity, animals were divided into four groups and all received HT treatment at 42 °C. Among the three formulations, IR of TSL was highest and NTSL was higher than ordinary injection then. Therapeutic effect of ordinary injection with HT is limited to HT cytotoxicity and any interaction between VB and HT; the NTSL cannot be triggered to release drug, its therapeutic effect with HT is limited to HT cytotoxicity, HT-induced liposomal delivery, and any interaction between VB that is released and HT. The TSL can be triggered to release drug quickly within short time (about 30 min), for this reason, beside HT cytotoxicity, HT-induced liposomal delivery, and any interaction between VB that is released and HT, the TSL's therapeutic effect with HT included HT-triggered liposomal drug release into tumor to increase local drug concentration to lead to apoptosis of tumor cells (Fig. 6). Thus, the combination of TSL with HT delayed tumor growth, increased therapeutic effect.

5. Conclusions

VB was successfully entrapped into liposomes by pH gradient loading method, and the entrapment efficiency was greater than 90%. In vitro drug release had thermosensitive characteristics, changed obviously with alteration of medium temperature.

In vivo, combining temperature-sensitive liposomes for VB with HT enhanced antitumor activity. The development of thermosensitive liposomes for VB provides an opportunity to improve the therapeutic index of this drug.

Acknowledgments

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