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Preparation, characterization and in vivo evaluation of 2-methoxyestradiol-loaded liposomes

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ABSTRACT

This study systematically investigated the intravenous injection formulation of liposomes loaded with 2-methoxyestradiol (2-ME), a poor water soluble anti-tumor drug. The objective of this study was to design passive targeting nanoliposome which could improve therapeutic efficacy and liver first pass effect. Based on the optimized conditions of single-factor and orthogonal design, 2-ME-loaded liposomes were prepared by the aether injection method. The formulated liposomes were found to be relatively uniform in size with a negative zeta potential. The average drug entrapment efficiency and loading were 85% and 8%, respectively. The overall targeting efficiency (TE^C) of the 2-ME-loaded liposomes was enhanced from 40.29% to 88.32% in the lung. The lung damage caused by liposomes was less severe than that by solution. These results indicated that 2-ME liposomes could mainly deliver the drug to the lungs and make the drug accumulate in the lungs, which changed the disposition behavior in vivo, decreased the toxic and side effects on other tissues and reduced the severity of damage to lungs following intravenous injection.

1. Introduction

Antiangiogenesis agent 2-methoxyestradiol (2-ME) is an endogenous metabolite of estrogen, which is synthesized in vivo by hydroxylation at the 2-position of estradiol, and subsequent catechol-O-methyltransferase mediated O-methylation. It inhibits growth of cancer cells (gastric, angiosarcoma, cervical, hepatocellular, colorectal, lung, prostate, breast, pancreatic, neuroblastoma, leukemia, multiple myeloma) (Barchiesi et al., 2006; Dobos et al., 2004; Garcia et al., 2006; Huh et al., 2006; Raobaikady et al., 2003; Sato et al., 2005). The actual mode of action of 2-ME has not been completely elucidated. However, the drug exerts its anti-tumor activity based on different mechanisms. Firstly, 2-ME in cancer cells binds to tubulin, and disrupts normal microtubule function through altering microtubule stability (D'Amato et al., 1994; Attalla et al., 1996, 1998; Mabjeesh et al., 2003; Mooberry, 2003a,b). Secondly, 2-ME induces cancer cells to undergo apoptosis such as through the mitochondrial intrinsic pathway by activation of c-Jun NH_2-terminal kinase (JNK) (Chauhan et al., 2003; Basu et al., 2006; Escuin et al., 2005). Thirdly, another most interesting feature of 2-ME is the inhibition of the pro-angiogenic transcription factor hypoxia-inducible factor 1-alpha, which leads to reduced expression of some tumor angiogenesis growth factors (e.g. vascular endothelial growth factor). Thus tumor angiogenesis is impaired by 2-ME (Sidor et al., 2005). At present 2-ME is in phase II clinical trials as a chemotherapeutic agent and has been orally administered to cancer patients (James et al., 2006), but it is highly lipophilic with very low water solubility, low bioavailability and the liver first pass effect (Ireson et al., 2004; Sweeney et al., 2005). Nanoparticulate systems targeted to macrophages have been proved to be a powerful approach for cancer. Following intravenous injection, nanoparticles have a natural tendency to accumulate in the organs that comprise the reticuloendothelial system (RES) (e.g. spleen, liver and lung) (Moghimi et al., 2001; Zhang et al., 2008). This natural passive targeting tendency could be utilized as a strategy for cancer therapy.

To the best of our knowledge, the effect of formulation parameters on 2-ME release profile from the most widely used biodegradable polymers, has not been studied so far, signifying the necessity of optimization of 2-ME/PC (phosphatidylcholine) formulations. Liposomes are well-recognized drug delivery vehicles, showing enhance the therapeutic activity of several anticancer drugs. In contrast to other delivery systems, such as micro-(nano)spheres, micro-(nano)particles, micro-(nano)capsules, micro-(nano)tubes or disks, liposomes exhibit some features, in terms of biocompatibility, biodegradability, low toxicity, entrapment abilities, variability of structures and physicochemical behaviors, which are very suitable for their application as delivery systems (Sun et al., 2008).

In this study, the design features of the system, its characterization and in vitro/vivo evaluation were described. The delivery system was characterized in terms of size, entrapment efficiency and drug loading. The aim of this study was to evaluate the effi-

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ciency that 2-ME/liposome nanoparticles can deliver 2-ME to lungs and also to avoid the liver first pass effect of oral adiministration and investigate the severity of lung damage.

2. Materials and methods

2.1. Materials and animals

2-ME (99.5% in purity) and letrozole (internal standard, 98.5% in purity) were home-made. Phosphatidylcholine (PC, injection grade) and cholesterol (CH) were purchased from Siwei (Zhengzhou, China). Poloxamer 188 (P188) was supplied by Shenyang Jiqi Pharmaceutical Co., Ltd. (China). Sephadex G 50 was obtained from Sigma–Aldrich Corporation (Milwaukee, WI, USA). Dialysis bags were provided from Sigma (molecular weigh cut-off, MWCO, 8000–14,000, USA). All reagents for high performance liquid chromatography (HPLC) analysis, including methanol and acetonitrile were of HPLC grade. Other chemicals used were of analytical grade.

Sprague–Dawley rats (200 ± 20) g and Kunming strain mice (20 ± 2) g were supplied by the Medical Animal Test Center of Zhengzhou University. The animals were used following the National Act on the use of experimental animals.

2.2. Preparation of 2-ME formulations

Liposomes were prepared by aether injection method (Batzri and Korn, 1973). In brief, required amount of PC, CH and 2-ME were dissolved in aether. An aqueous phase was prepared by dissolving P188 in doubly distilled water with 0.05 mol/L phosphate buffer saline solution (PBS, pH 7.4). The oil phase was dropped into the aqueous phase under rapid stirring at 1000 rpm (Stirrer Model HAM-1045, Tianjin Hengao Scientific Inc., China) for 30 min at 50 °C for dispersion. Then hydrated at 50 °C for 5 min. Liposomal dispersion was subjected to probe-ultrasonic (LTD JY92-II, Scientz Biotechnology Co., China) in an ice-bath. Homogeneous and slightly opalescent suspensions were produced. The effect of P188 on the stability of 2-ME/liposomes nanoparticle systems was investigated in detail.

Preparation of 2-ME solution: 2 mg/ml 2-ME solution was prepared by dilution of 2-ME stock solution in hydrogenated castor oil, alcohol and 0.5% saline solution mixture (1.5:0.5:8, v/v). 2-ME-loaded liposomes mixture was then freeze-dried as follows.

2.3. Freeze-drying of liposomes nanoparticle systems

A laboratory freeze drier (HERMLE CT/DW 110, Germany) was used to freeze-dry the nanoparticle systems. 10 ml of the liposome containing 20 mg 2-ME was suspended in distilled water containing cryo-protectant 4% trehalose–6% mannitol (w/v). The samples were frozen at −80 °C for 12 h followed by drying at −50 °C for 24 h under 20 Pa vacuum. The liposomes were rehydrated with 10 ml of 5% glucose solution before using.

2.4. Characterization of 2-ME-loaded liposomes

The morphology of 2-ME-loaded liposomes was observed by transmission electron microscopy (TEM) (JEM-1200EX, Jeol, Japan). Liposomes were diluted with distilled water and dropped on a formvar-coated copper grid (300-mesh, hexagonal fields) and air-dried for 1 min at room temperature after removing the excessive sample with filter paper. The air-dried samples were then directly examined under the TEM.

Mean vesicle size and zeta potentials of 2-ME-loaded liposomes suspension were determined using Zetasizer-Nano-ZS90 (Malvern Instruments, Malvern, UK). Analysis ($n = 3$) was carried out for 100 s at room temperature by keeping angle of detection at 90°.

2.5. Determination of entrapment efficiency and drug loading

2.5.1. Gel chromatography separation

A gel chromatographic column (1.0 cm × 25 cm) filled with Sephadex G-50 (Sigma Chemical Company, US) was used to separate liposome nanoparticle systems. 1 ml of 2-ME-loaded liposomes suspension was added to the column followed by elution with doubly distilled water. Free drug remained bound to the gel, while vesicles travelling the gel were collected from the first 25 ml and then 50 ml elution volume with alcohol–water (5:5) was free 2-ME.

2.5.2. HPLC analysis

Eluted liposomes were ruptured using sufficient volume of methanol and the drug content in the liposome dispersion was analyzed on Agilent 1200 series system (Agilent, USA). The HPLC conditions were as followings: ODS column (4.6 mm × 150 mm, 5 μm) (Agilent, USA) was used at room temperature. A mobile phase consisting of acetonitrile and phosphate buffer (pH5.5) (55:45, v/v) was at a flow rate of 1.0 ml/min, detection wavelength 285 nm, injection volume 20 μl. The drug entrapment efficiency (EE) and drug loading (DL) were calculated using Eqs. (1) and (2), respectively (Gómez-Hens and Fernández-Romero, 2006)

$$\mathrm{EE\%} = \frac{W_{\mathrm{total}} - W_{\mathrm{free}}}{W_{\mathrm{total}}} \times 100\% \quad (1)$$

$$\mathrm{DL\%} = \frac{W_{\mathrm{total}} - W_{\mathrm{free}}}{W_{\mathrm{lipids}}} \times 100\% \quad (2)$$

W_{free} is the analyzed weight of free drug in the supernatant; W_{total} is the analyzed weight of drug in the liposomes dispersions; W_{lipids} is the total weight of lipids.

2.6. In vitro release of 2-ME from liposomes

In vitro release of 2-ME from liposomes was performed using the dialysis bag diffusion technique (Avgoustakis et al., 2002), according to the dissolution test apparatus of Chinese Pharmacopoeia (2005 edition, paddle method). PBS (pH 7.4) – 0.4% SDS was used as dissolution medium. The dialysis bags (MWCO, 8000–14,000, Sigma) were soaked in deionized water for 12 h before use. 20 mg of the freeze-dried powder with 2-ME (equivalent to 1 mg 2-ME) were rehydrated in 0.5 ml of 5% glucose solution or 0.5 ml 2-ME solution (equivalent to 1 mg 2-ME) were placed in dialysis bags with two ends fixed by thread (the distance of the two nodes was 3 cm), respectively. Each bag was put into the flask containing 200 ml of dissolution medium. The flasks were placed into water bath at 37 ± 0.5 °C with paddle rotation at 100 rpm. While the solubility of 2-ME in the release medium was about 40 μg/ml, the maximum concentration of 2-ME in the medium was 5 μg/ml in this release experiment. So the sink conditions were assured. Aliquots of the dissolution medium (5 ml) were withdrawn at each time point (0, 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96, 120 h) and the same volume of fresh dissolution medium was added to the flask to maintain the constant volume. In addition, we also researched the accumulative release amount of 2-ME liposomes in other media, such as 1% Tween 80 and HCl (0.1 mol/l)–methanol (5:3). Drug concentrations in the dissolution medium were finally analyzed using the HPLC method as described previously. The release experiments were carried out in triplicates. The release rate was calculated using Eq. (3) and the results are expressed as

means ± standard deviation.

$$Q_n = C_n V_0 + \sum_{i=0}^{n-1} C_i V_i \qquad \text{release rate } (\%) = \frac{Q_n}{W} \times 100\% \tag{3}$$

Q_n is the accumulative drug release mass; C_n is the drug concentration of nth time point; V_0 is the total volume of the release medium; V_i is the volume of the withdrawn medium; C_i is the drug concentration of each time point; W is the total drug content of the release sample.

2.7. Chromatographic analysis conditions in vivo

HPLC 1200 system (Agilent, USA) with fluorescence detector was used to analize the concentration of 2-ME in vivo. The chromatographic conditions were as followings: The excited wavelength and emission wavelength were 285 and 325 nm, respectively. C_{18} column (150 mm × 4.6 mm, 5 μm) (Agilent, USA), a guard (C18, 10 mm × 4.6 mm, 5 μm) installed ahead of the analytical column, column temperature 30 °C, injection 20 μl, mobile phase consisting of 10 mmol/l potassium dihydrogen phosphate–acetonitrile–triethylamine (55:45:0.3, v/v/v, pH 3.0) was at the flow rate of 1.0 ml/min.

2.8. Pharmacokinetic studies in rats

Pharmacokinetics studies were performed as described elsewhere (Gao et al., 2006). Rats were randomly divided into two groups (5 per group). The rats used for this study were housed individually under normal conditions, and fasted overnight before experiment with free access to water. Group 1 was treated with 2-ME solution 1 ml while group 2 was administered with the freeze-dried powder with 2-ME (equal to 10 mg/kg 2-ME, 200 mg freeze-dried powder was suspended in 5 ml of 5% glucose solution) to obtain the same concentration. Each preparation was injected through the tail vein at the 2-ME dose of 10 mg/kg rats. 0.6 ml of blood samples were taken from the terminal retro-orbital bleeding at time (5, 10, 15, 30, 45, 60, 90, and 120 min) into micro-tubes containing sodium heparin as an anticoagulant, and centrifuged immediately (10 min, 3000 rpm) and stored at −20 °C until analysis. Liquid–liquid double extraction was performed prior to analysis by HPLC. Briefly, 200 μl of rat plasma was added to 8 μl letrozole (internal standard, 125 μg/ml), as internal standard and the mixture was extracted with 600 μl of ethyl-acetate vortexing for 2 min. Following centrifugation at 3000 rpm for 10 min, the organic phase was transferred to a glass tube and the solvent was evaporated under nitrogen stream at 40 °C. The dried sample was then dissolved in 100 μl mobile phase and 20 μl of the solution was injected into the column. HPLC conditions were described above. Pharmacokinetic parameters were evaluated using practical pharmacokinetic program version 3P97 (supplied by Chinese Pharmacological Society).

2.9. Tissue distribution study

Two groups of 60 rats were used for the distribution studies of 2-ME in vivo. One group was administered with 2-ME-loaded liposomes and another group was administered with 2-ME solution as control (10 mg/kg). The rats were anesthetized by inhalation of diethyl ether before blood sample collection. Five rats in each group were taken out randomly at each time point (0.25, 0.5, 1, 2, 4, 8 h). Blood samples were collected from the rats of each group after eyeball removal and placed into heparinized test tubes. The rats were sacrificed, dissected and each tested organ (heart, liver, spleen, stomach, kidney, lung and brain) was removed. Plasma was immediately separated by centrifugation (3000 rpm). Every organ sample was washed with saline solution and the redundant liquid was blotted using filter paper. Accurately weighted tissue specimen was placed in a homogenizing tube with double volume of acetonitrile–saline solution (1:1, v/v). 0.5 ml of each homogenate was mixed with 10 μl letrozole (internal standard, 125 μg/ml), then the extraction of 2-ME and letrozole was operated using 3 ml of ethyl-acetate. After vortexing for 3 min, the sample was centrifuged for approximately 20 min at 4000 rpm. The supernatant was collected and evaporated under nitrogen gas at 40 °C. The dry sample was reconstituted in 100 μl mobile phase for measurement of 2-ME using the same HPLC method mentioned above.

Overall targeting efficiency (TE^C) of 2-ME liposome were calculated and compared with that of 2-ME solution to evaluate the tissue targeting property of 2-ME liposomes. Overall targeting efficiency (TE^C) can be calculated according to Eq. (4):

$$TE^C = \frac{(AUC_{0-\infty})_i}{\sum_{i=1}^{n}(AUC_{0-\infty})_i} \times 100\% \tag{4}$$

In which the denominator refers to the sum total of drug exposure to all the tissues, including the target tissue. The data obtained from pharmacokinetic parameters were analyzed statistically by one-way analysis of variance and t-test using a statistical package for social sciences (SPSS version 11.0) software. Statistically significant differences were assumed when $p < 0.05$. All values are expressed as mean ± S.D.

2.10. Studies on the damage to mice lung

Eighteen Kunming strain mice (20 ± 2) g were divided into three groups at random with each group three male and three female ones. Groups A, B, C were administered pro i.v. with saline solution (0.2 ml/10 g), 2-ME liposomes (100 mg/kg) and 2-ME solution (100 mg/kg), respectively. After 48 h, the mice were anatomized, and the lungs were removed for histological analysis.

Table 1
The levels of experimental factors.

	A (w/w)	B (w/w)	C (v/v)	D (w/v)
1	5:1	3:1	3:1	0.05%
2	7:1	6:1	4:1	0.10%
3	10:1	12:1	5:1	0.20%

A, ratio of phosphotidylcholine to cholesterol (w/w); *B*, ratio of lipids to drug (w/w); *C*, ratio of H_2O to oil (v/v); *D*, concentration of poloxamer 188 (w/v).

3. Results and discussion

3.1. Preparation of 2-ME liposome

Pre-lyophilized 2-ME liposomes showed a mildly translucent liposome dispersion whilst lyophilized 2-ME liposome was white and amorphous powder. For all the experiments in the orthogonal design, the encapsulation efficiencies were within a range of 30–85%. Accordingly, we choose the optimized formulation to prepare the blank and 2-ME-loaded liposomes. The ratio of drug to PC (w/w), the ratio of PC to CH (w/w), the ratio of oil to water (v/v), and concentration of poloxamer 188 (w/v) were chosen as the most influential factors (labeled as *A*, *B*, *C* and *D* in Table 1). Taking the entrapment efficiency as an index, the four factors were investigated at three different levels. The L9 (3^4) orthogonal design was shown in Tables 1 and 2. The ranking of the four factors in this experiment was $B > D > C > A$, and the individual levels within each factor were ranked as: *A*, 3 > 1 > 2; *B*, 3 > 1 > 2; *C*, 1 > 3 > 2; *D*, 3 > 1 > 2. The optimal formulation should be $A_3B_3C_1D_3$ according to the analytical results using Orthogonality Experiment Assistant version 3.1 (Sharetop Software Studio).

Table 2
Orthogonal experiment design and drug entrapment efficiency EE (%) results.

	A	*B*	*C*	*D*	EE (%)
1	1	1	1	1	48.6
2	1	2	2	2	30.2
3	1	3	3	3	74.6
4	2	1	2	3	43.2
5	2	2	3	1	37.3
6	2	3	1	2	62.7
7	3	1	3	2	50.3
8	3	2	1	3	60.3
9	3	3	2	1	63.9
K_1	153.4	142.1	171.6	149.8	
K_2	143.2	127.8	137.3	143.2	
K_3	174.5	201.2	162.2	178.1	
K_{11}	51.1	47.4	57.2	49.9	
K_{12}	47.7	42.6	45.8	47.7	
K_{13}	58.2	67.1	54.1	59.4	
R	10.5	24.5	11.4	11.7	

A, ratio of phosphotidylcholine to cholesterol (w/w); *B*, ratio of lipids to drug (w/w); *C*, ratio of H_2O to oil (v/v); *D*, concentration of poloxamer 188 (w/v).

3.2. Characterization of 2-ME-loaded liposomes

TEM was conducted to investigate the morphology of liposomes, showing that the nanoparticles had spherical shapes. Vesicular structure was discernable (Fig. 1). The size distribution of the liposomes before and after freeze-drying was 167.6 ± 22.0 and 210 ± 25.0 nm, respectively. The negative zeta potential was −20.89 ± 2.0 mV. Liposomes presented a negative surface charge that would enhance the colloidal stability of the nanoparticles which have already been stabilized by poloxamer. Because poloxamer could efficiently adsorb at the nanoparticle surface and contribute to conceal recombination of nanoparticle (Santander-Ortega et al., 2006). The negative or neutral liposomes provide more effective barrier to plasma macromolecular protein adsorption and are easy to resuspend in blood (Mobed and Chang, 1998).

3.3. Entrapment efficiency and drug loading results

The experiments indicated that the ratio of drug to lipids (w/w) and the concentration of poloxamer 188 (%) were the most important factors affecting the drug entrapment efficiency. The entrapment efficiency of 2-ME-containing liposomes was 84.60 ± 0.73% before freeze-drying and 81.32 ± 0.61% after redissolved in 5% glucose and a drug loading 8 ± 2.1% were achieved in the preparation of 2-ME with the optimized formulation.

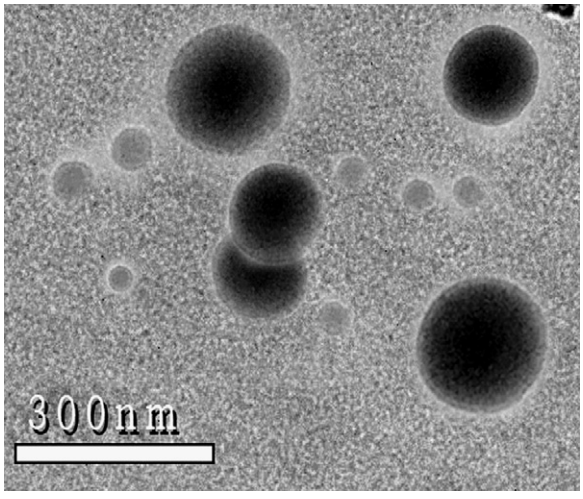

Fig. 1. Transmission electron micrographs of 2-ME-loaded liposomes.

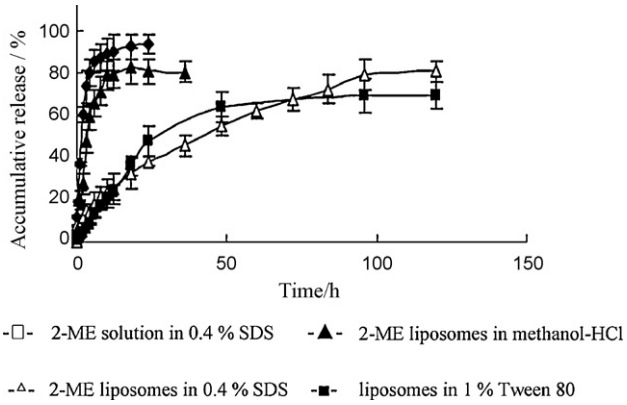

Fig. 2. In vitro release profile of 2-ME from 2-ME solution and 2-ME-loaded liposomes in different media at 37 °C (mean ± S.D., $n = 3$). (♦) 2-ME solution in 0.4% SDS; (▲) 2-ME liposomes in methanol–HCl; (△) 2-ME liposomes in 0.4% SDS; (■) liposomes in 1% Tween 80.

3.4. In vitro release of 2-ME from liposomes

The result of the accumulative release profile of 2-ME from liposomes is shown in Fig. 2. 2-ME release from stock solution was investigated as control. 2-ME was continuously released from the liposomes in 0.4% SDS-PBS (pH 7.4) for 120 h at 37 ± 0.5 °C. The initial fast release of around 20% of the drug from the 2-ME-loaded liposomes was observed in the first 5 h, which could be probably due to the portion of the drug that leaked out of liposomes and the unloaded drug (Glavas-Dodov et al., 2005). Subsequently, the release of drug from liposomes was slower and about 15% of the drug remained encapsulated in liposomes after 120 h of dialysis (Fig. 2). The delayed release might be attributed to the lipophilic 2-ME that was held by the small fragment of the liposomal membrane and the drug encapsulated in lipid membrane that released mainly through dissolution and diffusion from the lipid bilayer (C'ocera et al., 2000). Some lipophilic drugs in liposomes show a sustained release character. On the other hand, 2-ME with mother nuclear structure of steroids is a kind of steroid hormones, it can be binded by phospholipid bilayer and intermolecular interaction is different due to the phospholipid species and acyl chain-length (Perttu et al., 2006).

However, the release of 2-ME from liposomes in 1% Tween 80 was little faster than that in 0.4% SDS. In HCl (0.1 mol/l)–methanol solution (PBS as medium), 2-ME-loaded liposomes released rapidly about 80% within 10 h before reaching a plateau, which was similar with the release of 2-ME solution in 0.4% SDS. And the release of 2-ME from 2-ME solution was much fast, approximately 100% of drug dissolving in the release medium within 6 h (the solubility of 2-ME in three kinds of solution: HCl–methanol > Tween 80 > SDS). The in vitro release of a drug is an intention to define appropriate combinations of medium composition and an indirect method to predict its release action in vivo from a formulation. According to correlation level A: The in vitro release curve corresponds to the drug concentration of plasma in a 1:1 manner. The correlation analysis using SPSS program exhibited a relative good correlation in vivo/in vitro (HCl-methanol solution) (Anchalee and Pardeep, 2008) compared with other media aforementioned and the r is 0.95 ($p < 0.05$).

3.5. Pharmacokinetic studies in rats

In the pharmacokinetic experiment, the method of HPLC and letrozole as internal standard were used. The chromatograms showed stable baselines, as well as displaying good resolution among 2-ME, letrozole and endogenous material in plasma. The limit of detection was found to be 0.01 μg/ml in the present

Table 3
The pharmacokinetic parameters of 2-ME after intravenous administration of 2-ME solution and 2-ME-loaded liposomes in rats (mean ± S.D., $n = 5$).

Parameters	2-ME solution	2-ME-loaded liposomes
$AUC_{0-120\,min}$ (ng/ml min)	90115.9 ± 20,562	76052.31 ± 16,320
MRT_{0-120} (min)	103.79 ± 3.8	43.25 ± 3.3
CL/F (l/min/kg)	$1.11 \pm 0.14 \times 10^{-5}$	$1.31 \pm 0.24 \times 10^{-5}$
$T_{1/2\alpha}$(min)	8.6 ± 1.2	3.9 ± 0.7
$T_{1/2\beta}$(min)	119.7 ± 22.6	43.6 ± 7.7

conditions. The assay was linear in the range of 0.025–6.4 μg/ml. The regression equation was $A = 0.1406C + 0.0473$ ($r = 0.9995$, A was the ratio of area). The absolute recovery of 2-ME in rat plasma was 76.9–88.7%, and the relative recovery was 83.4–106.7%. Intra- and inter-day precision were <6.7%.

In vivo pharmacokinetic studies data indicated that the 2-ME solution followed a two-compartment model while 2-ME liposome suspension also followed two-compartment model with different pharmacokinetic parameters after i.v. administration of liposomes and solution at a dose of 10 mg/kg to rats (Table 3). Fig. 3 indicated that comparation in the curve of plasma concentration to time after injecting 2-ME liposomes and 2-ME solution. The comparison of other parameters within plasma showed that 2-ME liposomes had a lower $t_{1/2\alpha}$ and $t_{1/2\beta}$ than the respective value for 2-ME solution. These data indicated that encapsulation of 2-ME in conventional liposomes might promote its distribution in tissues, so the drug could rapidly act on therapeutic effect. The other important parameters, such as the mean residence time (MRT), AUC, CL/F, C_{max} were lower than 2-ME solution due to good biocompatibility and biodegradability of liposomes. In vivo, the release of drug from negatively charged liposomes in the presence of rat plasma may be due to the interaction of incorporated lipid compositions of liposomes with plasma components. Negatively charged liposome-entrapped drug was cleared more rapidly from the blood, resulting from the rapid uptake by the reticuloendothelial system to a greater extent. So the MRT from formulation was shorter than ME-solution (Chong-Kook et al., 1994).

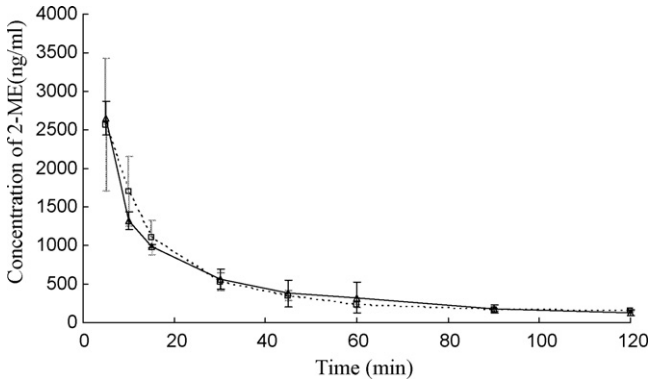

Fig. 3. Mean plasma concentration of 2-ME after intravenous administraton of 2-ME solution (△) and 2-ME liposomes (□) (mean ± S.D., $n = 5$).

3.6. Tissue distribution of 2-ME in rats

The limit of detection was found to be 0.01 μg/ml and the linearity of the method was demonstrated over the concentration range 0.025–6.4 μg/ml by analyzing plasma and various tissue standards in triplicate ($r \geq 0.9994$). The absolute recoveries of 2-ME in rat plasma and tissues were 73.42–85.70%, and the relative recovery was 92.64–109.16%. Intra- and inter-day precision were <9%, which were within the acceptance range. The 2-ME concentrations in the lung, liver and spleen of rats after i.v. injection of 2-ME-loaded liposomes increased from 4.521, 1.309, 1.832 μg/g to 118.763, 10.441, 7.809 μg/g, an enhancement of more than 26.27-, 7.98- and 4.26-fold, respectively, compared with the same dose of 2-ME solution (Table 4). The higher concentrations of 2-ME in lung, liver and spleen indicated that liposomes mainly accumulated in RES organs. These results demonstrated the passive targeting properties of liposomes towards RES organs (Fig. 4).

The 2-ME-loaded liposomes showed a good lung targeting property. The lung TE^C of 2-ME-loaded liposomes was increased from

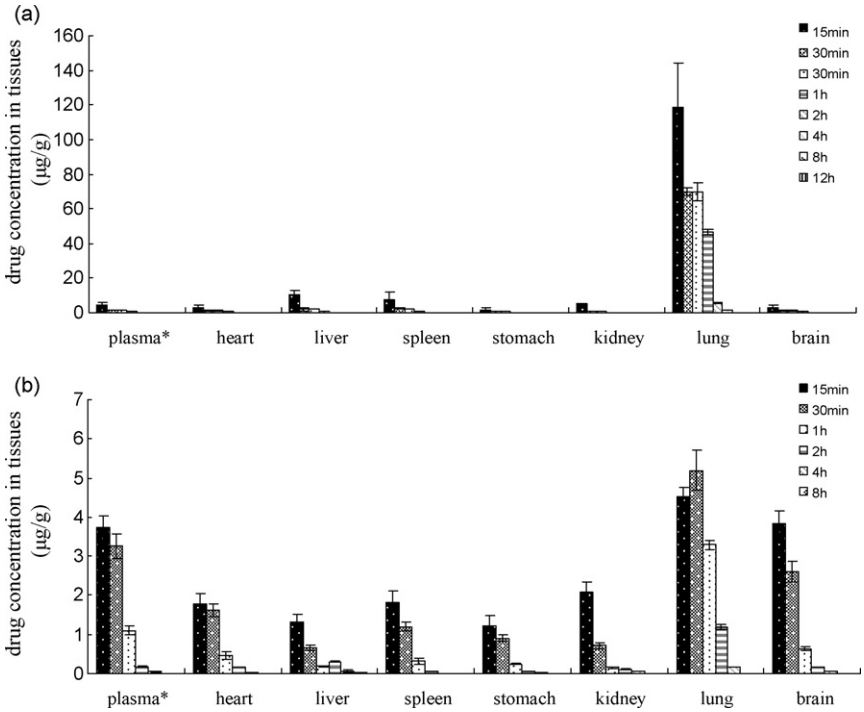

Fig. 4. The distribution of 2-ME in rats organs at different time points after i.v. administration of 2-ME-loaded liposomes (a) and 2-ME solution (b). *Unit of blood drug level: μg/ml.

Table 4
Targeting disposition of 2-ME after i.v. administration of 2-ME solution and 2-ME-loaded liposomes to rats (mean ± S.D., $n = 5$).

Tissue	2-ME-loaded liposomes		2-ME solution	
	C_{max} (μg/g)	AUC (μg/g h)	C_{max} (μg/g)	AUC (μg/g h)
Plasma [a]	4.367 ± 1.201	1.718 ± 0.556	3.734 ± 0.798	2.828 ± 0.821
Heart	3.133 ± 0.779	1.489 ± 0.484	1.775 ± 0.338	1.465 ± 0.534
Liver	10.441 ± 3.556	2.745 ± 0.834	1.309 ± 0.233	1.086 ± 0.356
Spleen	7.809 ± 2.145	2.279 ± 1.824	1.832 ± 0.387	0.947 ± 0.254
Stomach	1.813 ± 0.478	0.793 ± 0.235	1.218 ± 0.201	0.812 ± 0.135
Kidney	4.910 ± 1.445	1.465 ± 0.404	2.091 ± 0.621	0.865 ± 0.201
Lung	118.763 ± 35.668	88.416 ± 10.351	4.521 ± 0.932	6.913 ± 2.552
Brain	3.249 ± 1.125	1.202 ± 0.774	3.836 ± 0.874	2.244 ± 0.635

[a] Unit of blood drug concentration: μg/ml.

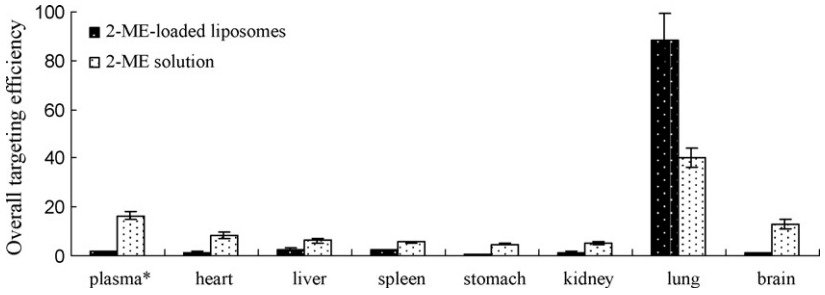

Fig. 5. Overall targeting efficiency (TE^C) of 2-ME-loaded liposomes and 2-ME solution (mean ± S.D., $n = 3$).

40.29% to 88.32% compared with 2-ME solution (Fig. 5). The total AUC in different tissues of 2-ME-loaded liposomes was 11.87-fold higher than that of 2-ME solution.

These results indicated that 2-ME-loaded liposomes could successfully target the lungs and correspondingly improve the efficacy of 2-ME to treat lung cancer. The reasons are mainly due to liposome encapsulating multiple molecules of drug to bind the receptors. Secondly, lowering the tensile force of alveolus surface, phospholipid can speed the spread of droplets of drug and the disaggregation of liposome so that drug on the alveolus surface can be absorbed rapidly. In addition, particles enwraped by non-ionic surfactants can accumulate to lung parenchyma (Lu, 2005).

2-ME is an endogenous metabolite of estrogen and there are specificity receptors of estrogens on rat lungs. Binding of estrogens in the cytosol from lungs of adult male rats was suppressible, dependent on incubation time and on protein concentration and was protein in nature. Suppressible binding of estrogens consisted of a high affinity site and showed evidence of positive co-operation. These results, together with the known effects of these hormones on certain lung functions, provide further evidence that lung is a target organ for estrogens (Benttarari et al., 1983). Because 2-ME solution is unimolecule, while 2-ME liposome encapsulate multiple molecules of drug to bind the receptors, the concentration of drug in lung is the highest.

Generally speaking, liposome composed of phospholipid is suitable for releasing drug into lung. By lowering the tensile force of alveolus surface, phospholipid can speed the spread of droplets of drug and the disaggregation of liposome so that drug on the alveolus surface can be absorbed rapidly. Also, it is reported that particles enwraped by non-ionic surfactants can accumulate to

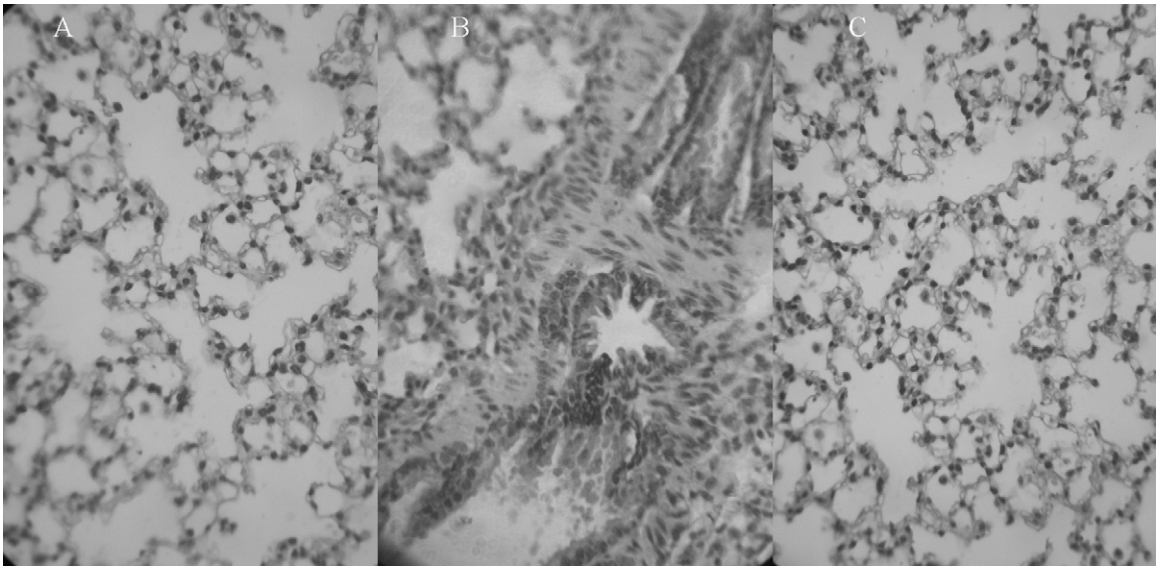

Fig. 6. Histological photographs of mice lungs after 2-ME delivery of (A) saline solution, (B) 2-ME solution and (C) 2-ME liposomes.

lung parenchyma. The higher concentration of 2-ME in lung in the present work may be caused by enwrapping 2-ME-liposomes with poloxamer188 which is a kind of non-ionic surfactants.

As for the effect of charge, theoretically, positively charged liposomes can be better used as carriers to deliver drug to cell membrane via electrostatic sorption, but there are also some reports about negatively charged liposomes, which is due to an endocytic mechanism for delivery (Kyung et al., 1992; Shailendra and Prahlad, 2008).

3.7. Histological observations of mice lungs in different groups

The histological result of these three groups was shown in the following photographs (Fig. 6). From Fig. 6B, it was found that the terminal bronchioles was next to lymphocytes infiltrating, inflammatory cells involved the bronchus mucoca, and smooth muscle around the terminal bronchioles were hyperplasic. In Fig. 6C, pathological changes were not observed, and there were not obvious changes compared with that of saline solution.

To assay the damage to the lungs after administrating 2-ME liposomes, histological analysis was conducted and the mice administered saline solution were as control. From the result, it was found that the lung damage of mice administered with the liposomes was significantly lower than that of 2-ME solution and the histological changes of mice given liposomes were not obvious compared with mice given solutions. In contrast, the tissue of mice which were injected with liposomes did not show significant difference from that of saline solution. The liposomes had better physiological adaptability.

4. Conclusion

In this work, we successfully incorporated the anticancer drug, 2-ME into liposomes nanoparticles for applying to intravenous administration and prepared its freeze-dried power to improve the stability of the drug. Compared with 2-ME solution, significantly improved the overall targeting efficiency to the lungs of rats. These results suggest that injectable liposomes of 2-ME may serve as passive targeting agents for lung cancer therapy. It has also been demonstrated that the liposomes are drug delivery systems which are able to modify tissue distribution, metabolism, and in vivo elimination as well as reduce the toxicity of free drugs (Ferrari, 2005). Further studies are needed to focus on the safety and efficiency of 2-ME-loaded liposomes to evaluate the potential clinical application value.

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