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Melittin liposomes surface modified with poloxamer 188: *in vitro* characterization and *in vivo* evaluation

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Received October 31, 2010, accepted November 16, 2010

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Pharmazie 66: 362–367 (2011) *doi: 10.1691/ph.2011.0327*

Melittin liposomes surface modified with poloxamer 188 were developed, and the effect of poloxamer 188 was investigated with regard to anti-cancer effect and vascular stimulation. Melittin liposomes surface modified with poloxamer 188 at different concentrations (0%, 2%, and 5%) were prepared using the adsorption method, followed by *in vitro* characterization, including entrapment efficiency, zeta potential, particle size, and morphology. Subsequently, the influence of repeated freeze-thawing on the liposomes was investigated, and the effect of poloxamer 188 on the repeated freeze-thawing process was explored. Vascular stimulation effects of MLT, and MLT liposome that surface coated with or without poloxamer were all studied. Pharmacokinetics of the different MLT preparations were determined and the anticancer activity of the MLT formulations was investigated. The particle size of the liposomes gradually increased with increasing poloxamer 188 content, while the entrapment efficiency did not change significantly. After the first freeze-thaw cycle, size and PDI were both markedly reduced, entrapment efficiency rose, and there was no significant change of zeta potential. The vascular irritation caused by MLT could be reduced to an extent by encapsulation in liposome, but not completely eliminated, while liposomes coated with poloxamer 188 can effectively abolish the phenomenon. Melittin liposomes with surface modified by poloxamer exhibit enhanced bioavailability, effective anticancer activity, and reduced side effects compared with melittin solution. Poloxamer plays an important role in melittin liposomes.

1. Introduction

Melittin (MLT), separated from bee venom, is an amphipathic, water-soluble peptide, consisting of 26 amino acids, and with 6 positive charges. It is the principal active component of bee venom accounting for 50% of its dry weight. MLT has been studied extensively due to its diverse bioactivity, such as antibacterial, anti-viral, anti-inflammatory, and anti-cancer effects (Terra et al. 2007). Especially in the last 20 years, it has been reported that MLT could kill various tumor cells directly (Liu et al. 2008; Wang et al. 2009), which has aroused increasing interest. There is evidence that the anti-tumor activity of MLT is higher than that of norcantharidin, garlicin, cinobufacin, mitomycin and vincristine (Ling et al. 2001). Therefore, MLT holds great promise as a new natural anticancer medicine. On the other hand, injection of MLT is associated with irritant reactions at the injection site. Treatment with MLT would result in erythema, pain and eventually edema, and even necrosis of vascular endothelial cells (DeGrado et al. 1982; Lee et al. 2004). Thus, the use of MLT therapy has been somewhat limited.

Micro- or nanoparticle-based drug delivery systems able to encapsulate therapeutic agents to avoid interaction with their environment are strong candidates to provide sustained, controlled, and targeted drug delivery to improve therapeutic effects and reduce side effects. Liposome are very sophisticated and promising particle-based drug delivery systems (Ranade 1989). However, several technological obstacles remain, such as drug leakage, brief blood circulation time resulting from rapid recognition, rapid sequestration of encapsulated drugs by macrophages, and lack of control over drug release rate. Thus, sterically stabilized liposomes (also known as long circulating liposomes) have often been used or considered for drug delivery (Woodle 1995; Liu et al. 2003). The stability of the liposomes can be enhanced by biocompatible polymer systems with covalent attachments, such as polyethyleneglycol (PEG). It has been reported that PEG-coated liposomes reduce mononuclear phagocyte system (MPS) uptake, and exhibit a slow release rate and long blood circulation time. It should be realized, however, that a covalently bonded PEG coating is not desirable for all steps in the drug targeting process, since it may hinder drug release and target cell interaction (Romberg et al. 2008). Attempts have been made to enhance the therapeutic efficacy of sterically stabilized nanoparticles by attaching a polymer to the liposome surface in a removable fashion (Immordino et al. 2006), allowing shedding (Romberg et al. 2008), to facilitate subsequent liposome capture by the cells.

Another alternative to steric stabilization of liposomes is achieved by the introduction of poly (ethylene oxide)-poly (propylene oxide)-poly (ethylene oxide) (PEO-PPO-PEO) nonionic triblock copolymers (Liang et al. 2005), which are

Table 1: Effect of different doses of poloxamer 188 on physicochemical properties of MLT liposomes (n = 3, mean ± SD)

Sample	Size (nm)	EE (%)
MLT-LIPO	140.0 ± 8.3	93.6 ± 1.5
MLT-LIPO-2%	193.4 ± 8.7	91.5 ± 0.8
MLT-LIPO-5%	264.7 ± 8.3	90.9 ± 1.9

known under the generic name poloxamer and the trademarks Pluronic®. Poloxamer consists of two hydrophilic PEO chains and a hydrophobic PPO chain, and is commercially available at low cost. Poloxamer 188 ($(PEO)_{79}(PPO)_{28}(PEO)_{79}$), a biocompatible triblock copolymer, approved by the FDA, has the lowest toxicity among poloxamer compounds.

Two preparation methods are used to introduce the poloxamer into liposomes. The first is based on incorporation, that is, copolymer molecules and phospholipid are mixed to form a film together, which is then followed by hydration and sonication steps. The second method is based on adsorption, with lipid vesicles being formed first, after which they are diluted with copolymer solution to the desired concentration (Kostarelos et al. 1999). Different methods may result in different modes of interaction of poloxamer with the lipid membrane: a) trans-membrane spanning, b) partial insertion; and c) adsorption (Liang et al. 2005; Kostarelos et al. 1999; Amado et al. 2009). No matter which mode is used, poloxamer can form a hydrophilic surface film which may help to reduce drug leakage in non-target regions.

Based on the above, in this paper we propose to develop MLT liposomes surface modified with poloxamer 188, and investigate their anti-cancer effect, and vascular stimulation, in order to study the effects of poloxamer, which is hypothesized to hinder MLT leakage and facilitate drug release at the targeted region.

2. Investigations and results

2.1. MLT liposome characterization

In this study, different concentrations of poloxamer 188 in the liposomes were used, to explore the influence of the concentration on the physicochemical properties of MLT liposomes (MLT-LIPO). The results are shown in Table 1.

As shown in Table 1, the size of the liposomes gradually increased as the amount of poloxamer 188 increased, while the entrapment efficiency (EE) did not change significantly. All the EE were high, though they fell slightly, suggesting that most of the MLT could readily be entrapped into the liposomes.

The three types of liposomes were regular spheres and were well rounded, with an obvious fingerprint and uniform bilayer lipid membrane. For example, the morphology of MLT liposomes modified with 5% poloxamer 188 (MLT-LIPO-5%) is shown in Fig. 1.

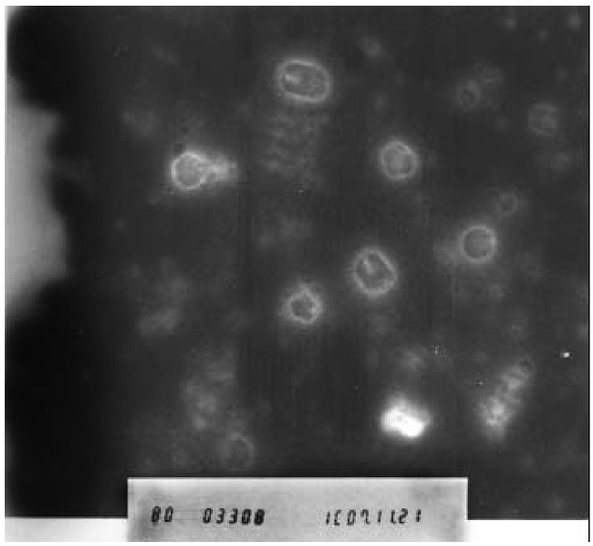

Fig. 1: Transmission electron photomicrographs of the MLT-LIPO-5% (×80000)

2.2. Influence of repeated freeze-thawing on the quality of MLT liposomes

The repeated freeze-thaw technique has been shown to be an effective method to enhance the stability of liposomes for water-soluble drugs (Castile and Taylor 1999; Stark 2010). herein this case we chose MLT-LIPO-5% as the model preparation to investigate the effect of freeze-thaw cycles on the characteristics of liposomes and the influence of poloxamer 188. The results are shown in Table 2.

The results show that the particle size and polydispersity index (PDI) of MLT-LIPO-5% were both markedly reduced after the first freeze-thaw cycle; EE was higher, while the change in zeta potential was not significant. The indices showed no significant change with an increased number of freeze-thaw cycles, indicating that the preparation process of MLT-LIPO surface modified with poloxamer 188 should include one freeze-thaw cycle.

The rupture of the phospholipid bilayer membranes is probably due to ice crystals formed during the rapid freezing process, while the exposed lipid would fuse together to re-form liposomes during the slow thawing process. During the process, the interaction between phospholipid and poloxamer was further strengthened. PEO chains were coated the surface while the PPO chains became inserted more deeply into the phospholipid bilayer membranes, possibly forming a trans-membrane spanning model. Thus, the size of the final liposomes was reduced, and the particle size distribution also became more uniform because of the lipid film reconstruction and the mutual fusion of liposomes of different sizes.

At the same time, the free amphiphilic MLT molecules could be further trapped in the liposomes, during the repeated freeze-thawing process, contributing to the increased EE of MLT.

Therefore, freeze-thaw technology could restructure and strengthen the interaction of lipid bilayers and poloxamer, further improving the stability and long circulating time of liposomes *in vivo*.

2.3. Vascular irritation of MLT liposomes

With the naked eye, purple veins could be seen on the right ear that received MLT solution (MLT-I), indicating that MLT causes strong vascular irritation. Compared with MLT-I, vascular stimulation MLT-LIPO is reduced, but slight purple coloration could still be seen. However, there was

Table 2: Changes of particle size, zeta potential and EE of MLT-LIPO-5% after repeated freeze-thawing (0, 1, 2, 3 times)

Cycle times	Size/nm	PDI	Zeta/mV	EE/%
0	264.7	0.458	–5.6	90.9
1	217.9	0.351	–7.2	98.9
2	216.8	0.363	–7.7	99.1
3	210.0	0.390	–6.0	97.6

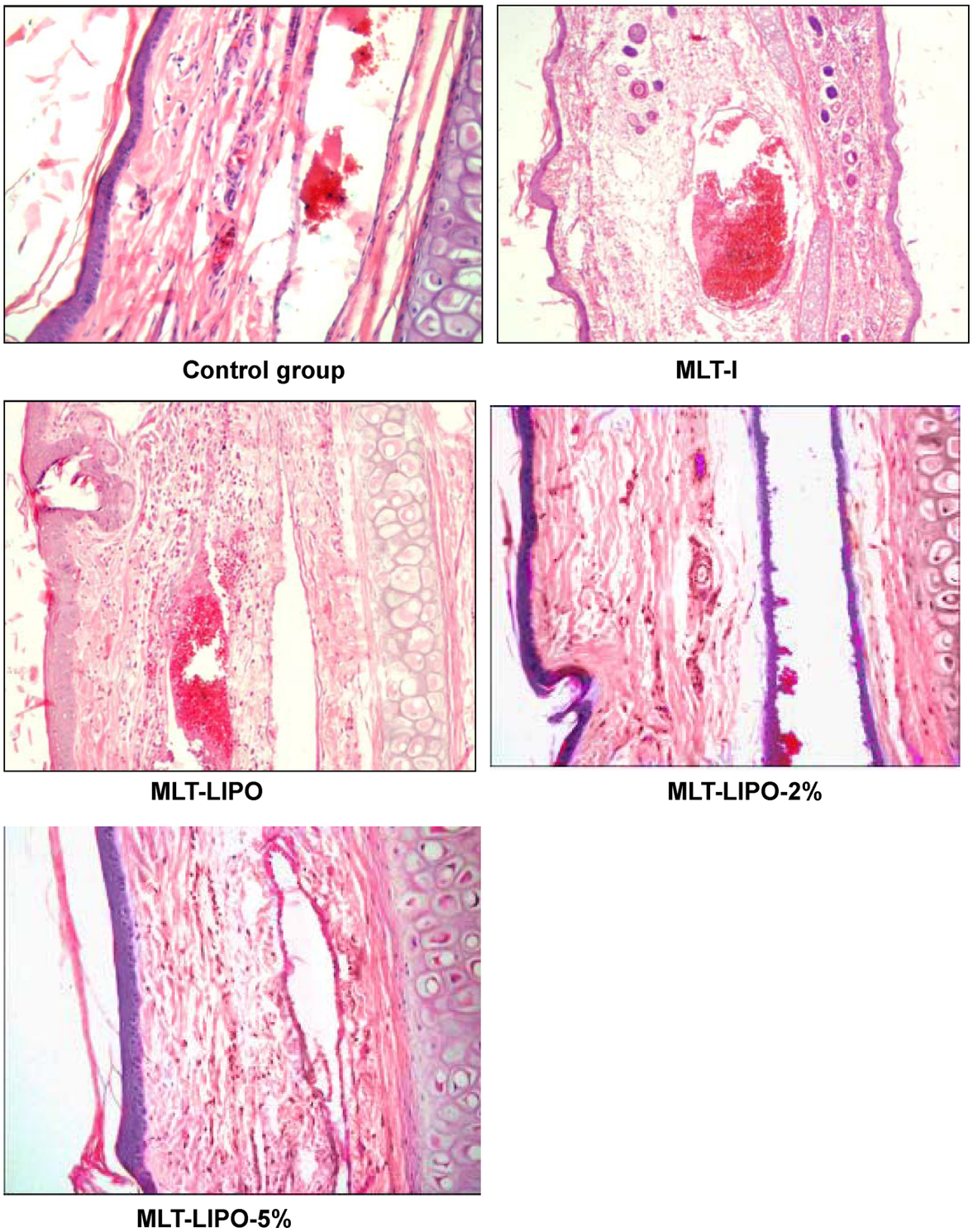

Fig. 2: Histopathological photograph of rabbit ear vein of MLT preparations and NS control groups (×100)

no difference between the groups treated with saline and MLT liposomes surface modified with poloxamer 188 (one is MLT-LIPO-5%, the other MLT-LIPO-2%, i.e, MLT liposomes modified with 2% poloxamer 188).

The same results can be seen from Fig. 2., Serious irritation appeared in the MLT-I group including swelling, thrombosis, edema of surrounding tissue and inflammatory infiltration. It was partially reduced in the MLT-LIPO group, but the vascular surrounding tissues presented slight bleeding and inflammatory infiltration. There was no significant difference between the saline control group and the MLT-LIPO-2% or MLT-LIPO-5% groups, none of which presented pathological changes.

The results show that the vascular irritation of MLT could be partially reduced but not completely eliminated through encapsulation in liposomes, while liposomes coated with poloxamer 188 can effectively prevent the phenomenon. This proves that

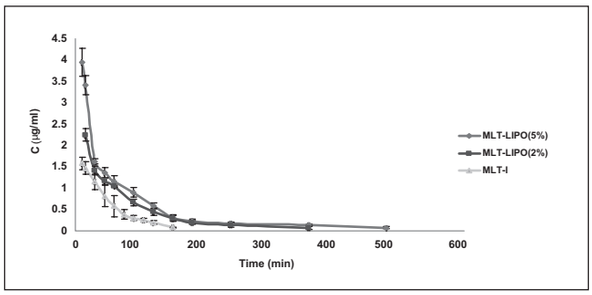

Fig. 3: The curve of plasma drug concentration–time after intravenous administration of MLT-I, MLT-LIPO-2%, MLT-LIPO-5% to rats, separately (n = 6)

liposomes surface modified with poloxamer188 could reduce the side effects and improve the safety of MLT.

2.4. Pharmacokinetic results in rats

In view of the improved safety of MLT liposomes surface modified with poloxamer 188, we chose MLT-LIPO-2% and MLT-LIPO-5% for the pharmacokinetic study, using MLT-I as a reference. Plasma concentration time curves of MLT-I, MLT-LIPO-2% and MLT-LIPO-5% are shown in Fig. 3.

The limit of quantification was reached at about 2.5 h for the MLT-I group, while detectable traces of MLT were still present in the serum 6 h after administration of MLT-LIPO-2%, and 8 h after MLT-LIPO-5%. The data demonstrate directly that the release of MLT in rats is slowed down by encapsulation in liposomes using poloxamer 188 to modify the liposome surface. Moreover, the release characteristics were related to the amount of poloxamer188 used.

Pharmacokinetic data analysis was performed using Kinetica 4.4 software. Some typical parameters, including: the area under the plasma concentration–time curve (AUC); total body clearance (CL); and mean residence time (MRT) are reported in Table 3.

As will be seen from Table 3, there were very significant differences between MLT-I and liposomal MLT surface modified with poloxamer 188. The AUC of MLT-LIPO-2% and MLT-LIPO-5% were 2.01 and 2.82 times that of MLT-I, respectively, the MRT of the two liposomes were 1.80 and 2.18 times, respectively, and CL in rats was decresed by 51.6% and 66.8% respectively. This further proves the long circulating time obtained with MLT liposomes surface modified with poloxamer188.

Comparison of the pharmacokinetic parameters of MLT-LIPO-2% and MLT-LIPO-5% shows that MRT was prolonged by 20.5%, CL was reduced by 31.5% and AUC was increased by 40.3% by increasing the amount of poloxamer 188 from 2% to 5%, and there was a significant difference between the two groups. The results support the finding that the entrapped MLT shows a longer MRT in rats, a much smaller CL and notably enhanced bioavailability, which shows a concentration-dependent relationship with poloxamer 188.

2.5. In vivo tumor growth inhibition study

The inhibitory ratios of MLT preparations are shown in Table 4. The results indicate that they all have a significant inhibitory effect compared with the negative control ($P < 0.01$).

Among the three MLT preparations given at the same dose, MLT-LIPO-5% had the greatest anti-tumor effect, compared with MLT-LIPO-2% and MLT-I, corresponding to the pharmacokinetic results. Moreover, the difference between MLT-LIPO-5% and MLT-I was significant using the t-test ($P < 0.05$). It may be the long circulating time and enhanced permeability and retention (EPR) effect of liposomes that contribute to MLT accumulation in the tumor region, which promotes the anti-tumor efficacy of MLT.

In addition, compared with the positive control, the MLT liposome groups all showed negative results for inhibition of weight gain, suggesting good safety. Though similar results were obtained in the MLT-I group, the tails of the mice became clearly rotted after 6 days injection.

In short, surface modification with poloxamer could help to enhance the therapeutic activity of MLT, especially when using a high concentration of poloxamer 188.

3. Discussion

MLT is a promising anti-cancer drug, but its adverse reactions such as pain at the injection site and vascular irritation limit its application. In order to overcome this obstacle, we developed some liposome preparations.

In this study, MLT liposomes surface modified with poloxamer 188 were prepared using the adsorption method, followed by one freeze-thaw cycle, which can strengthen the interaction between the lipid and poloxamer. After this the particle size of the liposomes was reduced, perhaps because the interaction changed from adsorption to trans-membrane spanning. The result of evaluation *in vivo* showed that the unmodified liposome can reduce the vascular irritation of MLT, but the phenomenon is not completely eliminated. This is due to lipid exchange between the phospholipids of the liposomes and alpha-lipoprotein (HDL) in the blood stream, followed by interaction with albumin, opsonin, antibodies, etc, resulting in the instability of the liposomes. Thus, the early leakage of MLT from the liposome causes slight stimulation.

The problem was solved by modification of the liposome surface with poloxamer 188. The hydrophilic PEO chains of poloxamer 188 cover the liposome, preventing the adsorption of plasma proteins on to the liposome surface, which decreases opsonification and improves the stability of the liposome in blood, so that leakage of MLT is notably reduced.

In addition, the hydrophilic film may help to circumvent the reticuloendothelial system (RES), giving a long circulating time. CL of MLT in rats is significantly decreased and MRT is prolonged, resulting in improved bioavailability.

On the basis of the enhanced therapeutic action and reduced side effects, we may conclude that poloxamer 188 plays an important role in MLT liposomes.

4. Experimental

4.1. Materials

4.1.1. Chemicals and reagents

Melittin (purity > 95%) was supplied from Anhui Baichun Bee Product Co., Ltd. (Anhui, China); Soybean phospholipids (Epikuron200) were generously donated by Degussa (Germany); Coomassie brilliant blue G-250 was obtained from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China); Cholesterol was purchased from Xinghua Biochemical Reagents Co., Ltd. (China); Triton X-100 and o-phenylenediamine (OPD) were from Sigma (USA); the BCA kit was obtained from Pierce (USA); Poloxamer 188 was from BASF (Germany); Anti-Apis Mellifera Venom antibody and goat anti-rabbit IgG-HRP antibody were purchased from Sigma (USA). Fetal calf serum (FCS) was provided by Sijiqing Bioengineering Co., Ltd. (Hangzhou, China); Cyclophosphamide (CTX) (200 mg/vial LN: 06060121) for injection was obtained from Jiangsu Hengrui Medicine Co., Ltd. (Jiangsu, China); All other reagents were of analytical purity.

4.1.2. Animals and cell line

Male Sprague-Dawley rats, weighing 200–250 g, and male ICR mice, weighing 18–22 g, were all purchased from Shanghai Silaike Laboratory Animal Co., Ltd. at China (Certificate No. SCXK 2003–0003).

Table 3: Pharmacokinetic parameters after i.v. 0.42 mg•kg^{-1} MLT-I, MLT-LIPO-2% and MLT-LIPO-5% on rats (n = 6)

Parameters	MLT-I	MLT-LIPO-2%	MLT-LIPO-5%
MRT/min	41.26 ± 2.50	74.46 ± 5.28***	89.75 ± 9.92***▲▲▲
CL/L·min^{-1}·kg^{-1}	$(4.46 \pm 0.64) \times 10^{-6}$	$(2.16 \pm 0.21) \times 10^{-6}$***	$(1.48 \pm 0.12) \times 10^{-6}$***▲
$AUC_{0\text{-}tn}$/min·μg·mL^{-1}	93081 ± 16304	187176 ± 17161***	262534 ± 10926***▲▲▲

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, MLT liposome vs MLT-I; ▲$p < 0.05$, △△$p < 0.01$, ▲▲▲$p < 0.001$, MLT-LIPO-5% vs MLT-LIPO-2%

New Zealand white rabbits weighing between 3 and 4 kilograms were bought from the Animal Center of China Pharmaceutical University.
Heps cells were obtained from the New Drug Screening Center of China Pharmaceutical University.
This experiment was conducted in accordance with the guideline issued by the State Food and Drug Administration (SFDA) of China. The animals were housed and cared for in accordance with the guidelines established by the National Science Council of the Republic of China.

4.2. Preparation of MLT liposomes

MLT liposomes with or without poloxamer 188 were all prepared using a freeze-drying and reconstitution technique. Concentrations of poloxamer 188 in vectors were far below the CMC (Tian et al. 2010; Maskarinec et al. 2002).
A mixture of soybean phospholipids and cholesterol (weight ratio = 8:1) was dissolved in the appropriate amount of dichloromethane in a round bottomed flask. The organic solvent was removed at 25∼30 °C by vacuum evaporation (RE52cs rotary evaporator, Shanghai Yali-wing Biochemical Instrument Factory, China). The vacuum was maintained overnight to remove any traces of solvent. Then the resulting lipid film was hydrated with PBS buffer (pH 8.0) at 37 °C, followed by sonication with an ultrasound probe (JY92 ultrasonic cell crusher, Ningbo Xinzhi Institute of Scientific Instruments, China) to form the blank liposome suspension. This was then mixed with MLT, which was dissolved in PBS buffer solution with an appropriate amount of lactose. After that, the mixture was extruded through 0.22 μm microporous membranes, and then freeze-dried to form unmodified MLT liposomes. The final concentration of MLT was 0.5 mg·mL^{-1}.
Poloxamer 188 modified liposomes were prepared using the adsorption method. The procedures were similar to the above, with the addition of 2% or 5% poloxamer 188 (poloxamer/phospholipids, weight ratio) to the hydration medium. Finally, two modified MLT liposomes, MLT-LIPO-2% and MLT-LIPO-5% respectively, were prepared.

4.3. Physicochemical characterization of MLT liposomes in vitro

4.3.1. Determination of entrapment efficiency of MLT liposomes

The EE of MLT liposomes was determined by an ultrafiltration-centrifugation technique. MLT liposomes suspension (300 μL) was added to the ultrafiltration device (OS100C33, MWCO = 100 kDa, Pall Corporation, USA), followed by centrifugation at 12000 rpm at 4 °C for 10 min. The lower part of the ultrafiltrate was collected. The amount of free drug (Mf) was determined by two-color colorimetric coomassie brilliant blue assay, while the total amount of MLT in the liposome suspensions (Mt) was determined by the bicinchoninic acid (BCA) method. The percentage of drug entrapped in the liposomes was calculated as follows: EE (%) = (Mt—Mf)/Mt × 100.

4.3.2. Size and zeta potential of MLT liposomes

1 mL of MLT liposome suspension was diluted in deionized water (1:4, v/v) followed by zeta potential and particle size measurement using a Zetasizer 3000HS (Malvern Instruments Ltd., UK).

4.3.3. Morphology assessment

The different liposome formulations were evaluated for their shape and vesicle type by transmission electron microscopy (H-7000, Hitachi, Japan). The diluted suspension was deposited onto copper grids coated with a porous polymer support. Excess sample was blotted away with filter paper. Phosphotungstic acid (1%) for 1 min was used as a negative stain.

4.4. The effect of repeated freeze-thawing process on the quality of modified MLT liposomes

Subsequent experiments used a repeated freeze-thaw procedure to investigate its influence on the EE, size parameters, PDI and zeta potential of freeze-thaw treated liposomes.
A sample of the finished suspension of MLT-LIPO-5% was frozen for 2 h at –70 °C, and then thawed for 15 min at 25 °C. The cycle was repeated once, twice and three times, respectively. EE, particle size, PDI and zeta potential of the liposomes was determined each time.

4.5. Evaluation of MLT liposomes in vivo

4.5.1. Vascular stimulation tests

Four rabbits were divided into 4 groups, each receiving an injection through the right marginal ear vein of 0.005 mg/mL of MLT, including sterile MLT-I, MLT-LIPO, MLT-LIPO-2% and MLT-LIPO-5%, while the left ear received 0.9% saline. The injected dose was 10 mL/kg, once a day, for four consecutive days.
After the final administration, the degree of redness, swelling and hyperemia or other irritation reactions in the injection region were observed for 48 h. The animals were then sacrificed. Along with the marginal ear vein from the injection point in the direction of the heart, continuous sections of 3 vascular vessels (each about 1 cm) were cut, together with peripheral tissues. All were fixed with 10% formaldehyde, followed by conventional dehydration and embedding in paraffin wax. Specimens were cut to 5 μm thickness. After hematoxylin-eosin staining, histological examination of the specimens was performed under a light microscope. Specimens of the control (0.9% saline) were treated by the same procedure.

4.5.2. Pharmacokinetic study in vivo

MLT determination by enzyme-linked immunosorbent assay (ELISA): Ninety-six-well plates were coated with a mixture of blood sample and an appropriate amount of coating buffer, followed by incubation for 12 h at 4 °C. Plates were washed three times with buffer solution (0.01 mol L^{-1}, pH7.4 PBST), and then blocked with 0.01 mol L^{-1} PBST containing 10% calf serum (pH7.4) for 2 h at 37 °C. After washing, 100 μL of primary antibody dilution buffer (pH 7.4 PBS) were added into each well for 2 h at 37 °C. Then, each well was incubated with 100 μL secondary antibody dilution buffer (0.01 mol L^{-1}, pH7.4 PBST containing 10% calf serum) for 2 h at 37 °C. After a final washing, the wells were incubated with OPD substrate buffer for 30 min at 37 °C, followed by addion of 50 μL H_2SO_4 (2 mol L^{-1}) to stop the reaction. The OD value was read at 492 nm using a microplate spectrophotometer (Thermo, USA).

Table 4: Inhibitory effect of MLT preparations on mouse transplanted by Heps (n = 10)

Group	Dose (mg·kg^{-1})	Weight (g) Before	Weight (g) After	Weight of tumor (g)	IE (%)
Saline	—	20.70 ± 1.34	27.67 ± 2.94	1.84 ± 0.20	0.00
CTX	3	19.30 ± 1.49	19.75 ± 4.74**	0.84 ± 0.13**	54.33
MLT-LIPO-5%	0.6	20.71 ± 1.60	27.57 ± 3.86	0.98 ± 0.21**▲	46.49
MLT-LIPO-2%	0.6	20.72 ± 1.52	26.85 ± 2.37	1.08 ± 0.18**	41.30
MLT-I	0.6	20.00 ± 0.67	25.60 ± 1.78	1.14 ± 0.09**	38.98

*$p < 0.05$, **$p < 0.01$, vs saline; ▲$p < 0.05$, MLT liposome vs MLT-I

Pharmacokinetic study: 18 male SD rats were divided randomly into three groups after fasting for 16 h to be administered MLT-I, MLT-LIPO-2% and MLT-LIPO-5%, respectively. Each rat received a dose of 0.42 mg/kg of the MLT sample through a femoral vein. After injection, blood samples of about 300 μL were taken from the retro-orbital plexus at various times, and subsequently centrifuged at 8000 rpm for 5 min at 4 °C. Then, 50 μL serum was added to an appropriate amount of coating buffer, and processed according to the above-mentioned ELISA to determine the concentration of MLT.
Statistics: Plasma concentration-time curves were fitted by Kinetica 4.4 pharmacokinetics software. The pharmacokinetics parameters obtained using the principle of statistical moments were analysed by the t-test.

4.5.3. In vivo tumor growth inhibition study

50 ICR mice were raised in an air-conditioned room under controlled lighting (12 h lighting/day) and were fed with standard laboratory food and water ad libitum.
Heps cells were subcutaneously inoculated into the right axillary fossa of the mice. After 24 hours, the mice were weighed and assigned randomly to 5 groups, each group containing 10 mice. Each group received one of the following treatment regimens: 0.9% normal saline (negative control group), 20 mg/kg cyclophosphamide (CTX, positive control group), MLT-I, MLT-LIPO-2% and MLT-LIPO-5% (dose of MLT 0.6 mg kg^{-1}). The treatments were administered i.v. into the tail vein at a frequency of once a day for a total of 6 days. The second day after the treatments, all mice were killed and weighed simultaneously, and then tumors were segregated and weighed. All the data were used for statistical analysis.

Acknowledgements: Jiangsu Key Laboratory of Carcinogenesis and Intervention, China Pharmaceutical University. This study was supported by the Major Project of National Science and Technology of China for New Drugs Development (No.2009ZX09310-004).

References

Amado E, Blume A, Kressler J (2009) Novel non-ionic block copolymers tailored for interactions with phospholipids. React Funct Polym 69: 450–456.

Castile JD, Taylor KMG (1999) Factors affecting the size distribution of liposomes produced by freeze-thaw extrusion. Int J Pharm 188: 87–95.

DeGrado WF, Musso GF, Lieber M., Kaiser ET, Kezdy FJ (1982) Kinetics and mechanism of hemolysis induced by melittin and by a synthetic melittin analogue. Biophys J 37: 329–338.

Immordino ML, Dosio F, Cattel L (2006) Stealth liposomes: review of the basic science, rationale, and clinical applications, existing and potential. Int J Nanomed 1: 297–315.

Kostarelos K, Tadros TF, Luckham PF (1999) Physical conjugation of (tri-) block copolymers liposomes toward the construction of sterically stabilized vesicle systems. Langmuir 15: 369–376.

Lee MT, Chen FY, Huang HW (2004) Energetics of pore formation induced by membrane active peptides. Biochemistry 43: 3590–3599.

Liang XM, Mao GZ, Simon Ng KY (2005) Effect of chain lengths of PEO–PPO–PEO on small unilamellar liposome morphology and stability: an AFM investigation. J Colloid Interface Sci 285: 360–372.

Ling CQ, HuangG XQ, Liu L (2001) The anti-tumor effect of melittin *in vitro*. Acad J Sec Mil Med Univ 22: 612–614.

Liu DZ, Hsieh YL, Chang SY, Chen WY (2003) Microcalorimetric studies on the physical stability of polyethylene glycol-grafted liposome. Colloid Surface A 212: 227–234.

Liu SJ, Yu M, He Y, Xiao L, Wang F, Song CC, Sun HS, Ling CQ, Xu ZH (2008) Melittin prevents liver cancer cell metastasis through inhibition of the Rac1-dependent pathway. Hepatology 47: 1964–1973.

Maskarinec SA, Hannig J, Lee RC, Lee KYC (2002) Direct observation of poloxamer 188 insertion into lipid monolayers. Biophys J 82: 1453–1459.

Ranade VV (1989) Drug delivery systems. 1. site-specific drug delivery using liposomes as carriers. J Clin Pharmacol 29: 685–694.

Romberg B, Hennink WE, Storm G (2008) Sheddable coatings for long-circulating nanoparticles. Pharm Res 25: 55–71.

Stark B, Pabst G, Prassl R (2010) Long-term stability of sterically stabilized liposomes by freezing and freeze-drying: Effects of cryoprotectants on structure. Eur J Pharm Sci 41: 546–555.

Terra RM, Guimaraes JA, Verli H (2007) Structural and functional behavior of biologically active monomeric melittin. J Mol Graph Model 25: 767–772.

Tian JL, Zhao YZ, Jin Z, Lu CT, Tang QQ, Xiang Q, Sun CZ, Zhang L, Xu YY, Gao HS, Zhou ZC, LiXK, ZhangY (2010) Synthesis and characterization of poloxamer 188-grafted heparin copolymer. Drug Dev Ind Pharm 36: 832–838.

Wang C, Chen TY, Zhang N, Yang MJ Li B, Lü X, Cao XT, Ling CQ (2009) Melittin, a major component of bee venom, sensitizes human hepatocellular carcinoma cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis by activating caMKII-tAK1-JNK/p38 and Inhibiting IκBα Kinase-NFκB. J Biol Chem 284: 3804–3813.

Woodle MC (1995) Sterically stabilized liposome therapeutics. Adv Drug Deliver Rev 16: 249–265.