International Journal of Pharmaceutics 370 (2009) 136–143

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Targeted delivery of methotrexate to skeletal muscular tissue by thermosensitive magnetoliposomes

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ARTICLE INFO

Article history:
Received 3 September 2008
Received in revised form
27 November 2008
Accepted 3 December 2008
Available online 7 December 2008

Keywords:
Thermosensitive
Magnetoliposomes
Targeted delivery
Liposome
Methotrexate

ABSTRACT

Thermosensitive magnetoliposomes (TMs) encapsulated with methotrexate (MTX) were prepared with 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and cholesterol by reverse-phase evaporation. Encapsulation efficiency of MTX and hydrophilic magnetite Fe_2O_3-glu, liposome particle size, ζ-potential, and *in vitro* and *in vivo* drug release were studied. More than 80% of loaded MTX was released from TMs within 30 min when the environmental temperature increased from 37 °C to 41 °C, while 60% of the drug was remained inside TMs for up to 24 h at 37 °C. Furthermore, the pharmacokinetics and tissue distribution study showed that TMs significantly increased the accumulation of MTX in the skeletal muscular tissue when exposed to an external constant magnetic field and heated to 41 °C compared to the absence of the magnetic field and heating. Therefore, the results in this study suggested that TMs prepared by reverse-phase evaporation can archive a good magnetic targeting effect and fast drug release in response to hyperthermia, which implies their great potential of application in cancer therapy.

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1. Introduction

There has been considerable interest in drug delivery for enhancing therapeutic efficacy and minimizing systemic side effects (Murphy et al., 2008; Tu and Kim, 2008; Zhu et al., 2008). For cancer chemotherapy, to avoid severe adverse effects, the drug molecules are expected to specifically kill the tumor cells, with only little or no adverse effect on normal tissues or cells. Significant progress has been achieved by targeted delivery of cytotoxic drugs to tumor area, which could effectively minimize the adverse effects of chemotherapy (Lee et al., 2007; Moro et al., 1997).

Liposomes which have a history of roughly 40 years (Bangham, 1993), have been proven to be a unique tool for studying both structural and dynamic aspects of natural membranes (Ozato et al., 1978; Sessa and Weissmann, 1970). Given their biocompatibility, biodegradability, low toxicity and immunogenicity, liposomes have attracted extensive attention during the past 30 years as pharmaceutical carriers of great potential (Klibanov et al., 1990). Although much progress has been archived in using liposomes in drug delivery, liposomal drugs have not yet led to a significant improvement in the clinical outcome (Batist et al., 2001; Harris et al., 2002) which is mainly due to their non-specific distribution caused by fast elimination from blood circulation and uptake by the reticulo-endothelial system (RES), primarily in the liver and spleen.

A number of strategies have been investigated to address this problem. The most successful technique was so-called long-circulating liposomes (LCL) (Allen et al., 1992; Klibanov et al., 1990). PEGylated liposomes with neutral hydrophilic shield effectively prevented non-specific protein binding and phagocytosis, and represented a successful example to prolong the circulation time in blood (Blume and Cevc, 1993). However, to increase the targeting efficiency, antibody (Singh et al., 1989), folate (Lee and Low, 1994), transferring (Ishida et al., 2001), cancer cell homing peptide (Myrberg et al., 2008), arginine–glycine–aspartic acid (RGD) (Du et al., 2007) among others, have been reported to be used as ligands to modify liposomes for targeting to specific tissues and organs. Besides the surface modification chemistry, physicochemical methods represent another promising way to control the delivery and targeting processes for liposomal drugs. The liposomes made with these physicochemical elements could respond to a stimulus within the micro-environment of diseased sites and release the loaded drug by a second internal or external stimulus, i.e. temperature (Needham et al., 2000), light (Kostarelos et al., 2005), magnetic field (Nobuto et al., 2004) or pH (Roux et al., 2003).

Magnetic liposome or magentoliposome is a liposome which incorporates either magnetic materials in its aqueous core (Sabate et al., 2008; Zhang et al., 2005) or magnetized polymers within its

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doi:10.1016/j.ijpharm.2008.12.003

lipid bilayer structure (Kamaly et al., 2008; Leclercq et al., 2003), and enables targeted delivery of therapeutic molecules to a specific site exposed in a magnetic field. Since firstly prepared in 1990s (Kiwada et al., 1986a,b) magentoliposomes have been well developed in recent years (De Cuyper et al., 2007; Kullberg et al., 2005; Zhang et al., 2005).

Thermosensitive magnetoliposomes (TMs) combine magnetic targeting delivery and temperature sensitive drug release, where triggered release of drugs can be realized when the environmental temperature goes beyond the phase-transition temperature of liposomal components (Masuko et al., 1997; Viroonchatapan et al., 1998, 1996). The drug release rate and extent can be adjusted by altering the phase-transition temperature which is determined by both type and molar ratios of lipid bilayer components (Ben-Yashar and Barenholz, 1989).

TMs have been shown to be effective at the presence of an external magnetic field in an in situ mouse liver model (Viroonchatapan et al., 1996) and in the tumor bearing mice (Masuko et al., 1997; Viroonchatapan et al., 1998). Skeletal muscle which belongs to peripheral compartment has no affluent blood flow. Therefore, it is difficult for drug molecules to accumulate in skeletal muscle by conventional carriers which are distributed through the circulation system. Furthermore, targeting delivery of therapeutic molecules to skeletal muscle in whole body level has rarely been reported. The goal of this study is to prepare TMs with methotrexate (MTX) as a model drug, which can result in both site-specific delivery and triggered release of methotrexate to mouse skeletal muscle. The liposomal formulation was carefully optimized based on the *in vitro* drug release, PK and tissue distribution of MTX in mice. Our data showed that MTX TMs enhanced the drug release and accumulation in the skeletal muscular tissue when exposed to a magnetic and hyperthermia field.

2. Materials and methods

2.1. Materials

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) was purchased from Lipoid (Ludwigshafen, Germany). MTX was purchased from Huzhou Outlook Pharmaceutical Co., Ltd. (Huzhou, China). The reference standard of methotrexate (HPLC grade, purity 99.8%) was prepared by the Department of Analytical Chemistry in China Pharmaceutical University (Nanjing, China). Theophylline (HPLC grade) was purchased from Xinhua Pharmaceutical (Zibo, China). Single-crystal magnetic nanoparticles (γ-Fe_2O_3-Glu, 6 g/L, 10 nm) were prepared by Nanjing University (Nanjing, China). Cholesterol and heparin were purchased from HuiXing Biochemistry Reagent Limited Company (Shanghai, China). Dialysis tubing (MWCO 8,000) was purchased from Huamei Biological Company (Shanghai, China). Methanol and water were HPLC grade. All other chemicals used were commercially available and of analytical grade.

2.2. Preparation of TMs

TMs were prepared by a modified reverse-phase evaporation method as described before (Zhu et al., 2006). Briefly, DPPC and cholesterol (67:33, mass ratio) were dissolved in 4 mL of chloroform and diethyl ether mixture (4:3, v/v) in a round-bottom flask. γ-Fe_2O_3-Glu (1 mL, 6 g/L) and MTX solution (1 mL, 3 mg/mL) were added dropwise and the mixture was stirred for about 40 min in an ice bath (0 °C) until a stable W/O emulsion formed. Then, this emulsion was applied onto a rotary evaporator and the organic solvent was removed slowly under reduced pressure (260–400 mmHg) at 43 °C. The coarse TM suspension was formed by phase reversion during solvent removal.

After reverse-phase evaporation, non-encapsulated magnetite was removed by centrifugation at 5000 rpm for 20 min twice (Viroonchatapan et al., 1996). Thereafter, the particle size of liposome was reduced by sonication for 5 min and uniformed by repeatedly passing through 0.8 μm filter membrane. Finally, TM suspension was dialyzed against sterile water with MWCO 8000 overnight to remove free MTX.

2.3. Determination of particle size and ζ -potential

After preparation of MTX TMs, the liposome suspension was diluted with distilled water (pH 6.0). The particle size and ζ-potential were determined at 25 °C by dynamic-light scattering using a Mastersizer-2000 Particle Size Analyzer (Malvern Instruments, UK). A polydispersity index (PDI) which ranges from 0.0 to 1.0, was used to estimate the polydispersity of the liposome dispersion.

2.4. Determination of MTX encapsulation efficiency

To determine the encapsulation efficiency, non-encapsulated MTX was separated by dialysis and quantified by UV spectrometry at its maximum absorbance wavelength of 306 nm. Briefly, the coarse TMs suspension (1 mL) was dialyzed (MWCO 8000) against 50 mL of distilled water. Complete equilibrium of non-encapsulated MTX across the dialysis membrane was assumed when the concentration of MTX in the dialysis medium did not increase for 2 h. After equilibrium, the concentration of non-encapsulated MTX was equal to that of free MTX which can go through dialysis membrane. The total MTX was measured by directly dissolving TMs in ethanol. The encapsulation efficiency was calculated by the following formula:

$$\text{Encapsulation efficiency}\,\% = \frac{(\text{MTX}_{\text{Total}} - \text{MTX}_{\text{Free}})}{\text{MTX}_{\text{Total}}} \times 100\%.$$

2.5. Quantification of encapsulated iron oxide

The encapsulated Fe_2O_3 in TMs was measured by UV spectrometry. Briefly, 1 mL of MTX TMs was diluted 2-folds with water and 1 mL of the diluted suspension was transferred to a volumetric flask followed destruction of liposomal structure with ethanol. Thirty milliliters of water was added and the pH of the solution was adjusted to 6–8 by hydrochloric acid (1 M). Then 1 mL of 10% hydroxyamine hydrochloride, 2 mL of 0.15% orthophenanthroline and 5 mL of sodium acetate (1 M) were added. The mixture was diluted with water to the final volume of 50 mL and measured by UV spectrometer at the wavelength of 510 nm. The entrapment of iron was shown as micrograms of Fe_2O_3 per milliliter TM suspension.

2.6. Temperature dependent MTX release

One milliliter of MTX TM suspension was placed in a dialysis tubing (MWCO 8000). To determine the thermosensitivity, dialysis was performed against 50 mL of saline at the temperature ranged from 30 °C to 44 °C. The sample was collected at 20 min post incubation at each temperature and released MTX (C_T) was quantified by UV spectrometer. MTX TMs was measured by directly dissolving in ethanol as 100% release (C_∞). MTX release was calculated as:

$$\text{MTX release}\,(\%) = \frac{C_T}{C_\infty} \times 100\%.$$

To mimic the *in vivo* drug release, dialysis was performed against 50 mL of saline at 37 °C for 24 h and at 41 °C for 1 h respectively. UV

spectrometer was used to quantify the released methotrexate (C_t) at different time point in each sample. Methotrexate release was calculated as:

$$\text{Methotrexate release}\,(\%) = \frac{C_t}{C_\infty} \times 100\%.$$

2.7. Quantification of MTX by HPLC

All of the biological samples were determined by Agilent 1200 series HPLC system, which was carried on a Diamonsil C_{18} reversed-phase column (6.0 mm × 150 mm, 5 μm) with a detection wavelength of 306 nm using the isocratic elution consisted of methanol and 10 mM sodium dihydrogen phosphate solution (25:75, v/v) at a flow rate of 1.0 mL/min at 25 °C.

2.8. Sample processing

100 μL of theophylline (1 mg/mL) and 40 μL of perchloric acid (70%) were added to 100 μL plasma sample. For tissue sample preparation, 0.3 g of tissue was weighted and homogenized in 1 mL of saline. Then, 400 μL of tissue homogenate was added with 100 μL of theophylline and 40 μL of perchloric acid.

The sample mixture was thoroughly vortex-mixed for 2 min followed by centrifuge at 14,000 rpm for 4 min. Then, 500 μL of the supernatant was transferred to a fresh tube and 3 mL of ethyl acetate and isopropyl alcohol (10:1, v/v) were added, followed by vortex-mixing and centrifuge at 6000 rpm for 4 min. The supernatant was blown dry with a stream of nitrogen at 50 °C. The residue was re-dissolved in 50 μL of 0.1 M sodium hydroxide and an aliquot of 20 μL of this solution was injected into the HPLC system for analysis (Wang et al., 2006).

2.9. Pharmacokinetic and iodistribution study

Mice (20 ± 2 g) were obtained from the Laboratory Animal Center of China Pharmaceutical University (Nanjing, China). All mice were specific pathogen free animals bred in the Laboratory Animal Center of China Pharmaceutical University. The research protocols were approved in advance by the Institutional Animal Care and Use Committee of China Pharmaceutical University.

In total 140 mice were randomly assigned to 4 groups, 35 mice per group. Group 1 was administered with MTX solution via tail vein injection. Groups 2, 3 and 4 were administered with MTX loaded TMs via tail vein injection. For group 3, the hind limbs of mice were exposed in a 0.2 T magnetic field produced by a permanent magnet immediately after administration. For group 4, the hind limbs of mice were exposed in both magnetic field and water bath at 41 ± 0.5 °C immediately after administration. For group 2, mice were not exposed in either magnetic field or warm water bath after administration. The dose of MTX was 10 mg/kg in all experimental mice. At 5, 15, 30, 60, 90, 120, 240 and 360 min post administration, 0.5 mL of blood was collected by retro-orbital sampling. Then, mice were sacrificed and the major tissues including liver, spleen, kidney, and skeletal muscle of the hind limbs were collected. The tissues were washed by saline and blotted dry. After weighted, the samples were stored at −20 °C for assay.

2.10. Pharmacokinetic calculation

Non-compartmental pharmacokinetic analysis was performed using software 3P87 (Administration of Health, Beijing, China). Briefly, the elimination rate constants (k) were determined by linear regression on the logarithmic transformation of the last four data points of the concentration–time curve. The elimination half-life ($t_{1/2}$) was calculated by the following equation: $t_{1/2} = 0.693/k$. The area under the concentration–time curve up to the last time point (t) (AUC_{0-t}) was determined using the trapezoidal rule. The $AUC_{0-\infty}$ values were calculated by adding the value of C_t/k to AUC_{0-t}.

2.11. Statistical analysis

Data were expressed as the mean ± standard deviation (SD). The difference in methotrexate concentrations among different experimental groups was determined by one-way ANOVA. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Preparation of MTX TMs

Several preparation methods had been investigated, which included hand shaking method, sonication and ethanol injection. All methods gave either low encapsulation efficiency (drug or iron) or unstable multilamellar liposomes with large particle size and distribution. Reverse-phase evaporation method is the effective method for preparing liposomes with water soluble drug. We reported MTX magnetoliposomes with high encapsulation efficiency using the modified reverse-phase evaporation method in the previous work (Zhu et al., 2006). Organic solvents including ethyl ether, ester acetate and chloroform were investigated to form a stable W/O emulsion at 0 °C, which can decrease evaporation of solvents and stabilize the emulsion. TMs were formed by phase transition from W/O to O/W at 43 °C during the organic solvent evaporation.

Hydration medium also influences the drug entrapment efficiency. In this study, we found ionic strength strongly affected methotrexate entrapment during hydration process. 5% mannitol, 5% glucose, and distilled H_2O gave more than 60% of MTX entrapment compared to only about 40% using saline as the hydration medium.

MTX is insoluble in pure water and organic solvents, such as ethanol, ether and chloroform. To prepare TMs by reverse-phase evaporation, MTX was solubilized by forming a salt with sodium hydroxide at the concentration of 3 mg/mL.

After TMs formation, the non-encapsulated nano-sized iron particles were separated from liposomes by centrifuge. The particle size of TMs was usually more than 1 μm after centrifuge. Viroonchatapan et al. had reported that 75% of 5-FU TMs was distributed in the range of 1.32 ± 0.12 μm post centrifugation (Viroonchatapan et al., 1996; Viroonchatapan et al., 1997). The large particle size was not safe for i.v. injection. Therefore a short-time sonication at low power was used to break the aggregate and reduce the particle size in case of the large leakage of liposomal contents. Finally, the particle size distribution was narrowed by repeatedly passing through 0.8 μm filter. The dialysis tubing with MWCO of 8000 was efficient for free MTX (MW 454.45) to be removed from particulate liposomes.

3.2. Particle size and ζ-potential

After preparation, the particle size, PDI and ζ-potential of TMs were determined and found to be 581 ± 64 nm, 0.12 ± 0.01 and 4.20 ± 0.09 mV, respectively. There were more than 90% of the liposomes below the particle size of 630 nm.

3.3. Quantification of encapsulated iron

Free form Fe^{2+} was formed by dissolving Fe_2O_3 in acidic solution followed by pH adjustment to 6–8. Color reaction was performed by the formation of red Fe (O-ph)$_3$ between chromogenic agent,

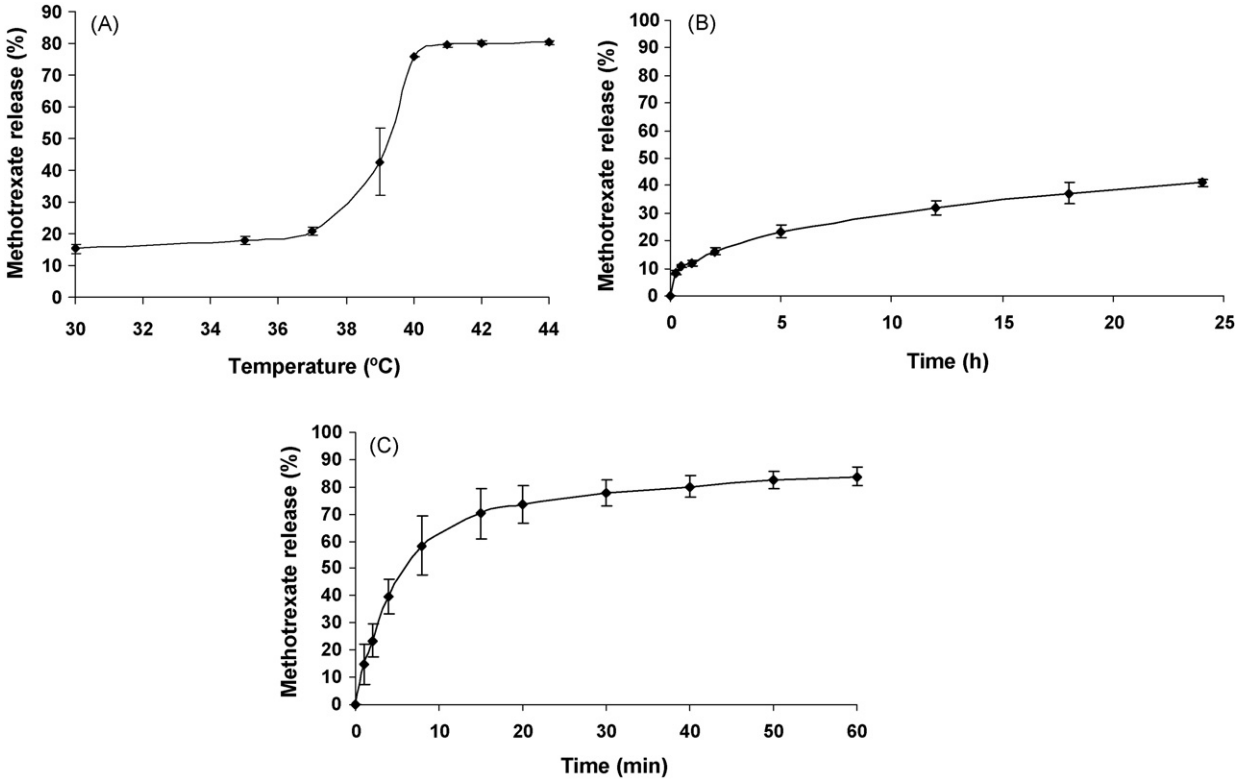

Fig. 1. *In vitro* drug release of MTX TMs. (A) The percentage of MTX release after incubation of MTX TMs at each temperature for 20 min. (B) The percentage of MTX release at 37 °C up to 24 h. (C) The percentage of MTX release at 41 °C within 60 min. The values were expressed as mean ± SD ($n = 3$).

orthophenanthroline (O-ph), and Fe^{2+} in the presence of hydroxylamine hydrochloride and sodium acetate. Fe $(O\text{-ph})_3$ was stable in the solution up to 6 h based on UV absorbance (RSD < 0.8%) and sufficient for colorimetry to detect. This method was effective and sensitive. The regression formula is $A = 0.0691C + 0.0061$ ($r = 0.9999$), linear range: 1.92–12.8 μg/mL of Fe_2O_3. After removal of non-encapsulated iron oxide particle by centrifuge, the concentration of the encapsulated Fe_2O_3 was 626.1 μg/mL of liposome suspension.

3.4. Encapsulation efficiency of methotrexate

The mass ratio of 67:33 between DPPC and cholesterol was used to formulate the MTX TMs. After removal of the free MTX, the concentration of the encapsulated drug was determined by UV spectrometry. The MTX TMs had a high average encapsulation efficiency of 61.4 ± 1.8%. The regression formula is $A = 0.0144C + 0.0104$ ($r = 0.9999$), linear range: 10.12–60.72 μg/mL.

3.5. In vitro drug release

The *in vitro* drug release was studied at different temperatures (Fig. 1A) and different time points (Fig. 1B and C). When the temperature increased from 39 °C to 41 °C, the drug release significantly increased from below 20% to more than 80% (Fig. 1A). Result in Fig. 1B showed that the drug release was very slow and incomplete (40%) even up to 24 h at 37 °C. However, at 41 °C, the drug release reached 70% within 10 min and continued to release drugs up to 90% within 60 min (Fig. 1C). The release of MTX from the TMs was showed to be temperature dependent.

3.6. In vivo assay method validation

The interference by endogenous substances was assessed by inspection of chromatograms derived from processed blank and mouse samples spiked with MTX. MTX had a retention time of about 10.5 min, while internal standard theophylline had a retention time of about 8.0 min under the same chromatographic condition. No interference peak was found at the retention time of theophylline and MXT.

The HPLC method exhibited good linear response over the selected concentration ranges by linear regression analysis for plasma, liver, spleen, kidney, and skeletal muscle samples. Accuracy, intra- and inter-day precisions were evaluated over five validation days. The relative standard deviation (RSD) values for intra-day precision and accuracy were, respectively, in the range of 2.0–8.8 and 99.92–102.4%, whereas the corresponding inter-day values were 2.6–11 and 99.48–102.2%. The results revealed good precision and accuracy. The extraction recovery determined for MTX was shown to be acceptable and reproducible. The mean recoveries of the three concentration levels were within the range of 76.9–92.5%, whereas the RSDs were within the range of 1.1–8.7% for plasma, liver, spleen, kidney, and skeletal muscle samples.

3.7. Pharmacokinetics and biodistribution studies

After intravenous administration of MTX solution and MTX liposomes in the presence of either magnetic field or heating, or both of them, the concentrations of MTX in plasma, liver, kidney, spleen and skeletal muscle were determined by HPLC. The concentration versus time profiles of these tissues were shown in Fig. 2. The corresponding pharmacokinetic parameters in plasma and tissues were listed in Table 1.

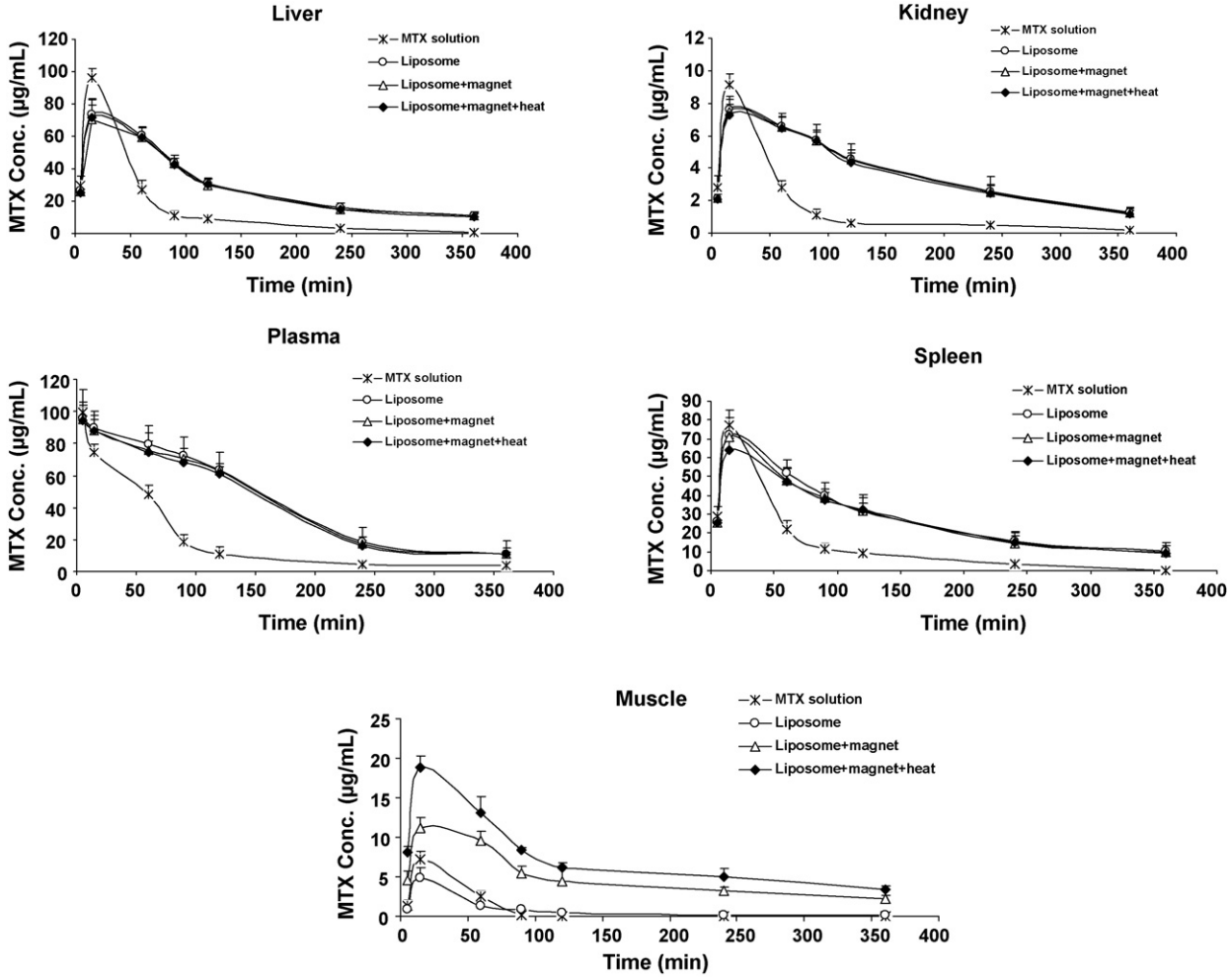

Fig. 2. Plasma and tissue concentration–time curves after intravenous administration of MTX solution and TMs. 140 mice were randomly assigned to 4 groups, 35 mice per group. Group 1 was administered with MTX solution via tail vein injection. Groups 2, 3, and 4 were administered with MTX loaded TMs via tail vein injection. For group 3, the hind limbs of mice were exposed in a 0.2 T magnetic field produced by a permanent magnet immediately after administration. For group 4, the hind limbs of mice were exposed in both magnetic field and water bath at 41 ± 0.5 °C immediately after administration. For group 2, mice were not exposed in either magnetic field or warm water bath after administration. The dose of MTX was 10 mg/kg. Sampling was scheduled at 5, 15, 30, 60, 90, 120, 240 and 360 min post administration. The values were expressed as mean ± SD (n = 4).

Table 1
Pharmacokinetic parameters of MTX in mouse plasma and tissues after intravenous administration of MTX solution and TMs at the same dose (n = 4).

	Group	Cmax (μg/mL)	$t_{1/2}$ (min)	MRT (min)	CL (mL/min)	AUC_{0-t} (μg min/mL)
Plasma	Solution	99.37	119.2	71.9	0.026	7,073.46
	Liposome	95.62	96.9	108.5	0.011	16,261.40
	Liposome + magnet	94.94	105.6	107.6	0.012	15,790.70
	Liposome + magnet + heat	94.18	104.6	106.4	0.012	15,316.42
Liver	Solution	96.19	57.1	59.9	0.037	5,425.92
	Liposome	73.86	159.5	116.8	0.015	10,745.63
	Liposome + magnet	70.37	120.4	116.6	0.016	10,408.36
	Liposome + magnet + heat	71.35	119.4	116.2	0.016	10,388.45
Spleen	Solution	77.33	45.0	63.5	0.043	4,681.26
	Liposome	72.05	122.4	118.2	0.017	10,200.44
	Liposome + magnet	70.99	130.5	117.6	0.017	9,885.58
	Liposome + magnet + heat	64.13	121.0	118.8	0.018	9,633.21
Kidney	Solution	9.13	131.6	69.8	0.349	533.56
	Liposome	7.60	127.5	127.7	0.124	1,373.23
	Liposome + magnet	7.47	125.3	127.3	0.127	1,351.33
	Liposome + magnet + heat	7.22	122.7	126.8	0.131	1,321.59
Muscle	Solution	7.18	14.8	29.7	0.643	309.77
	Liposome	4.90	79.4	62.0	0.710	270.67
	Liposome + magnet	11.20	235.5	128.3	0.081	1,724.20
	Liposome + magnet + heat	18.86	280.7	127.9	0.050	2,619.13

With MTX solution treatment, C_{max} in liver, spleen, kidney and plasma were much higher than those of other treatments. Application of the external magnetic field significantly increased C_{max} from 4.90 to 11.20 μg/mL in the skeletal muscle. Combination of heating and magnet treatment further increased the C_{max} in the skeletal muscle significantly from 11.20 to 18.86 μg/mL. The $t_{1/2}$ in plasma and kidney with MTX solution treatment was much longer than those of other treatments. However, in live, spleen and skeletal muscle, the $t_{1/2}$ of all liposome treatments were much longer than that of MTX solution treatment. Especially in skeletal muscle treated with both magnet and heating, $t_{1/2}$ was increased up to 3.5-folds compared to liposome treatment alone and up to 20-folds compared to MTX solution treatment. MRT of all liposome treatments in all the tissues were longer than that of MTX solution treatment. Among the liposome treatments in the tissues except skeletal muscle, there was no significant difference on MRT. In skeletal muscle, the application of magnet or both magnet and heating significantly increased the MRT more than 2-folds compared to the liposome treatment group, while there was no significant difference between magnet treatment and both magnet and heating treatments.

Comparing CLs in the plasma and tissues except skeletal muscle, administration of MTX TMs regardless of additional treatments significantly decreased the clearance compared to those of MTX solution treatment, while there was no significant difference among different additional treatments with the injection of MTX TMs. In skeletal muscle, administration of MTX TMs treated with both magnet and heating reduced CL to 0.050 mL/min, which was the lowest clearance compared to 0.081 mL/min of magnet treatments and 0.710 mL/min of liposome treatments alone.

Administration of MTX TMs regardless of additional treatments had much higher AUC_{0-t} compared to those of administration of MTX solution in plasma and the tissues except skeletal muscle. Application of additional treatments on mouse limbs slightly decreased AUC_{0-t} in these tissues. However, in skeletal muscle, AUC_{0-t} was significantly increased almost 10-folds with the treatment of both magnet and heating compared to the absent of magnet and heating.

4. Discussion

Methotrexate, which is a potent anti-neoplastic agent and has very low accumulation in skeletal muscle after i.v. injection (Chen and Chiou, 1982), was used as a model drug to evaluate the targeted delivery efficiency and drug release of thermosensitive magnetoliposomes in this study. In addition to the *in vivo* behavior of MTX, the skeletal muscle is belonged to the anatomically clearly defined peripheral compartment in the PK models because of its low blood flow rate. Therefore, the use of conventional liposome which prefers to accumulate in liver, spleen and lung depending on its particle size, surface charge and ligand, will not be useful for the targeted delivery of MTX to skeletal muscle. Therefore, in this study, we designed a thermosensitive magnetoliposome which effectively combined the targeting property of magentoliposome with the temperature dependent drug release into TMs.

Hyperthermia is a type of treatment in which body tissue is exposed to high temperatures (i.e. 41 °C) to damage and kill cancer cells, or to make cancer cells more sensitive to the effects of radiation and certain anticancer drugs, generally by increasing blood flow, membrane permeability, local metabolism and drug efficacy (Bicher et al., 1980). Hyperthermia is always used in combination with other forms of therapy, such as radiation therapy, chemotherapy, and biological therapy to increase their effectiveness. The TMs utilize both the magnetic field guided targeted delivery and local hyperthermia triggered drug release, which are easy to be controlled and adjusted regarding clinical demands.

It has been shown that there is a maximal release of liposome contents at a temperature close to the gel-to-liquid crystalline transition (Papahadjopoulos et al., 1973). It is also known that the phase transition is affected by the liposome composition including the types of lipids, the ratio between lipids and additives such as other types of surfactants (Ben-Yashar and Barenholz, 1989). In order to obtain maximal release of loaded drug at 41–42 °C from liposomes which are stable at 37 °C, the phase transition should start at the temperature higher than 40 °C. Commonly the lipids with high transition temperature can be used to formulate thermosensitive liposomes, which include 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC, T_m 41.5 °C) (Weinstein et al., 1979), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC, T_m 55 °C) (Weinstein et al., 1979), and hydrogenated soy phosphatidylcholine (HSPC, T_m 55 °C) (Gaber et al., 1996; Papahadjopoulos et al., 1991). In this study, we prepared themersensitive magnetoliposome using DPPC and cholesterol which gave the fast and significant drug release at 41 °C, but very slow and incomplete drug release at 37 °C up to 24 h (Fig. 1).

Magnetite which was known as the ferrimagnetic particles, such as Fe_3O_4 or γ-Fe_2O_3, have been explored extensively (Sabate et al., 2008; Zhang et al., 2005). It is an ideal magnetic material for the core of magnetoliposomes. Unlike ferromagnetic materials, magnetite has a lower stray magnetic field intensity which may minimize the potential health risks posed by magnetic fields (Chignell and Sik, 1998), and a lower chance of agglomeration due to magnetostatic interactions during preparation and storage of liposomes. In order to further reduce agglomeration, γ-Fe_2O_3 was stabilized by glutamic acid, a metal chelating agent, which is able to form chemical bonds with the metal ions. In this study, γ-Fe_2O_3-Glu was used as the magnetic material because of its small particle size, biocompatibility and non-toxicity (Sousa et al., 2001).

To study the *in vivo* behaviors of magnetoliposomes, the permanent magnet could be implanted in the disease tissue such as tumor mass (Kubo et al., 2000, 2001) or placed externally around disease site (Viroonchatapan et al., 1996; Zhang et al., 2005). Obviously, the latter method is more convenient because the application time and site can be fully controlled by external manipulation according to the clinical demands. Also this method has no lesion to the mice during the experiment. Based on these considerations, in this study we used a permanent magnet (0.2 T) as an external magnetic force which was placed on the skin of the right hind leg of the mouse. MTX TMs exhibited excellent magnetic responsiveness with more than 2-folds of C_{max} and 5-folds of AUC_{0-t} compared to those at the absence of the magnet (Table 1). Using this delivery strategy, the targeting delivery efficiency was significantly enhanced; meanwhile the drug release time of MTX was prolonged as evidenced by significantly prolonged CLs in the target tissues (Fig. 2 and Table 1).

Intravenous injection of doxorubicin magnetoliposomes produced a 3–4-fold higher C_{max} in the tumor tissue in the presence of a dipole external electromagnet compared to significant reduction in other tissues (Nobuto et al., 2004). RGD-anchored magnetoliposomes increased the drug level in brain for about 6-folds compared to free drugs, and 7.6-folds in comparison to nonmagnetic formulation after 4 h, while liver uptake was significantly reduced (Jain et al., 2003). In our study, 3.85-folds increase of C_{max} and 9.68-folds increase of MTX accumulation (AUC_{0-t}) in skeletal muscle compared to the absence of magnet were observed, while significant reduction in other tissues was not seen. The reason may be the low blood flow in skeletal muscle which caused the difficulty in MTX accumulation and makes the negligible amount of MTX in skeletal muscle even in the presence of magnetic field. But, this does not mean MTX TMs lost its function since the temperature dependent drug release could achieve a sustained release with the terminal half-life of 280.7 min compared to about 120 min in liver, spleen and kidney. Furthermore, our data suggested that MTX TMs may be

more powerful in the tumor chemotherapy because the tumor has a rich vessel system and high blood flow rate which will magnify the accumulation of chemotherapeutics. Such targeted drug delivery for anticancer agents would provide clinical advantages compared to conventional methods.

5. Conclusions

In conclusion, we have developed the MTX loaded TMs which successfully carried its contents to skeletal muscle in the presence of external magnet, and released MTX quickly and completely in a temperature dependent manner in a mouse model. The properties of targeted delivery and temperature-dependent drug release could be easily controlled and adjusted by changing the magnetic field, the application site and liposome formulation. Based on our present data, TMs would be more efficient if it were used in cancer chemotherapy.

Acknowledgement

This work was supported by a grant ZKX0308 from Nanjing Medical Bureau, Nanjing, China.

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