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Research Paper

Lyophilized Paclitaxel Magnetoliposomes as a Potential Drug Delivery System for Breast Carcinoma via Parenteral Administration: *In Vitro* and *in Vivo* Studies

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Purpose. The study reports *in vitro* and biological evaluation of lyophilized negatively charged paclitaxel magnetic liposomes as a potential carrier for breast carcinoma via parenteral administration.
Methods. Paclitaxel in magnetoliposomes were extracted by centrifugation and quantified by high-performance liquid chromatography (HPLC). Biological properties were studied using pharmacokinetics, *in vivo* distribution and cytotoxicity assays, as well as a mouse model of EMT-6 breast cancer.
Results. Pharmacokinetic studies showed that encapsulation of paclitaxel in magnetoliposomes produced marked difference over the drug in Cremophor EL/ethanol pharmacokinetics, with an increased $t_{1/2\beta}$ 19.37 h against 4.11 h. For *in vivo* distribution, paclitaxel concentration of lyophilized magnetoliposomes in the tumor was much higher than that of lyophilized conventional liposomes or Cremophor EL/ethanol, whereas in heart it was much lower than the latter two formulations via s.c. and i.v. administration. Lyophilized paclitaxel magnetic liposomes showed more potency on the therapy of breast cancer than other formulations via s.c. and i.p. administration.
Conclusions. The current study demonstrates that paclitaxel magnetoliposomes can effectively be delivered to tumor and exert a significant anticancer activity with fewer side effects in the xenograft model.

KEY WORDS: *in vitro*; *in vivo*; magnetoliposomes; paclitaxel; parenteral administration.

INTRODUCTION

Paclitaxel, belonging to the taxane class of anticancer agents, perhaps is the most important chemotherapeutic agent against cancer over several decades (1–3). Paclitaxel inhibits cell proliferation by inducing a sustained mitotic block at the metaphase/anaphase boundary (4). It promotes the polymerisation of stable microtubules, inhibits their disassembly, and profoundly affects a number of key cellular functions that depend on the turnover of tubulin. In clinical trials, paclitaxel has been successfully used for the treatment of ovarian, breast, lung, and head/neck cancers and AIDS-related Kaposi sarcoma (5,6). The main toxicities for the drug are neutropenia, peripheral neuropathy, and hypersensitivity reactions. They have been observed relative to the formulation (5,7). Paclitaxel, which is extremely insoluble in water as well as in other vehicles commonly used in parenteral dosage, is currently formulated in a vehicle composed of 50:50 (v/v) blend of Cremophor EL (polyethoxylated castor oil) and ethanol. Cremophor EL has shown to induce histamine release and severe allergic reactions (8).

Given the drawbacks of the current paclitaxel formulation, there is a strong rationale for reformulating paclitaxel using a safer and better tolerated vehicle than Cremophor EL/ethanol formulation. Many attempts have been made to achieve good selectivity to targeted tumor cells by preparing specialized carrier. Among these, liposomes are the most studied colloidal particles thus far applied in medicine and in particular in antitumor therapy. Paclitaxel was the first taxane to be encapsulated in liposomes, with promising results both *in vitro* and *in vivo* (9–12). After i.v. or i.p. administration, paclitaxel-liposome formulations were much better tolerated than paclitaxel and showed antitumor activity similar to that of the free drug in Cremophor EL/ethanol modality. These colloidal carriers are, however, subjected to opsonic phagocytosis by circulating phagocytes and by macrophages of liver and spleen (13,14). One of the strategies to overwhelm this problem is to magnetize the drug-loaded carrier so that it can be guided to and retained at the target site with the help of an external magnetic field of appropriate strength. Retention of magnetic carrier at target site can apparently delays reticuloendothelial clearance and neodisposition of the contained drug. Magnetic liposomes have been investigated for targeted drug carrying potentials (15–20).

To improve the water solubility of paclitaxel and explore the best type of paclitaxel liposomes, lyophilized paclitaxel-loaded negatively charged and magnetic liposomes were evaluated for their preferential presentation to the tumor under neoplastic condition, and enhanced drug level in tumor and plasma, as well as decrease of drug uptake in heart, liver, and spleen, was found. The aim of this work was therefore to

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further investigate parenteral paclitaxel loaded magnetoliposomes and to fully study their physicochemical and biological properties.

MATERIALS AND METHODS

Materials

Hydrogenated soybean phosphatidylcholine was from Lucas Meyer GmbH (Hamburg, Germany). Cholesterol, dicetylphosphate (DCP), and α-tocopherol (TOC) were from Sigma Chemical Co. (Poole, UK). Paclitaxel was from Zishan New Technology Company Ltd. (Shanghai, P.R.China). Taxol (paclitaxel in Cremophor EL/ethanol, 5 ml: 30 mg per vial) was obtained from Taiji Pharmaceutical Co. (Sichuan, P.R. China). Dixipan control was from National Institute for the Control of Pharmaceutical and Biologic Products (Peiking, P.R. China). Both ultrafine magnetite (Fe_3O_4 in the matrix of 0.02 mol/L of Tris-HCl buffer, mean diameter 50 nm) and external magnetic field (circular permanent magnet tablet, Sm_2C_{01T}, Φ10 mm × 2 mm, 2000 G) were from Southwest Institute of Applied Magnetics of China (Chengdu, P.R. China). Methanol and acetonitrile were of high-performance liquid chromatography (HPLC) grade from Tedia Co. (Fairfield, OH, USA). All other reagents and solvents used were of analytical grade.

Preparation of Lyophilized Magnetoliposomes

Paclitaxel magnetoliposomes were prepared by the reverse evaporation method. Briefly, hydrogenated soybean phosphatidylcholine, cholesterol, dicetylphosphate, and α-tocopherol were dissolved in 5 ml of diethyl ether with molar ratio of 62:31:7:2.5. Paclitaxel 4.2 mg was dissolved in 0.5 ml of chloroform and mixed with above mixture. Then, 4.5 ml of diethyl ether was added, and the solvent was evaporated from the mixture in a rotary flash evaporator at 30°C ± 0.5°C. The lipid film obtained was dissolved in 30 ml of diethyl ether and introduced with 5 ml of ultrafine magnetite (50 nm), placed into a ice-water bath sonicator until one-phase liquid formed. Then the liquid was evaporated in high vacuum to form gel and water-like suspension in turn. The evaporation continued for 10 min, and then the suspension was flushed with nitrogen, sealed, and placed in an ice-water bath sonicator for one-half hour. Eight percent lactose was added as a supporter, and lyophilized for 24 h. The lyophilized powder was hydrated with 0.02 mol/L of Tris-HCl buffer before use and was used in all the following tests unless mentioned particularly.

Similar to lyophilized paclitaxel magnetoliposome preparation, the lyophilized conventional liposomes were prepared. The only difference was that no ultrafine magnetite was added in the preparing process.

Micrographs were taken with a transmission electron microscope for the morphologic analysis, and the particle size and distribution of liposomes were measured with laser particle analyzer (Malvern mastersizer 2000, Worcestershire, UK).

Quantification of Paclitaxel and Determination of Entrapment Efficiency

Reversed-phase HPLC methods were used for the analysis of paclitaxel content. Drug content analysis was performed using a Symmetry C18 column (5 μm, Kromasil ODS, Bohus, Sweden) equipped with a C18 guard column at 25°C with a mobile phase containing a mixture of methanol, acetonitrile, water (30/40/32, v/v/v) at a flow rate of 1 ml/min. Sample injection volumes were 20 μl and paclitaxel detection was performed using UV detector at a wavelength of 227 nm. Peak area was recorded and processed on a C-R7A Shimadzu interface (21,22).

Entrapment efficiency of paclitaxel in magnetoliposomes was determined by a modified centrifugation method (9,21,23). Briefly, 1 ml of paclitaxel magnetoliposomes was added to 4 ml of 5% ethanol and the mixture was subjected to centrifugation at 22,000 × *g* for 5 min to separate free paclitaxel from the magnetoliposome entrapped drug. The suspension, which contained free paclitaxel, was transferred into a suitable tube and extracted with diethyl ether, then evaporated to dryness under nitrogen. The residue was reconstituted with 0.5 ml of methanol. Dixipan 100 μl was added into the sample as internal standard and vortex-mixed for 1 min, and then the sample was analyzed for paclitaxel concentration using the HPLC method described previously. The following equation was used to calculate the entrapment efficiency.

$$\%\ \text{Drug entrapment efficiency} = \text{Drug}_{\text{encapsulated}}/\text{Drug}_{\text{total}} \times 100\% = (\text{Drug}_{\text{total}} - \text{Drug}_{\text{free}}) \times 100\%$$

Here, the amount of initially added drug was regarded as that of total drug (24) because the drug loss in the preparation process could be negligible (data not shown).

Different amounts of standard paclitaxel were added into 1 ml of blank liposomes and treated with the aforementioned method, and then the recoveries of paclitaxel were determined and calculated.

Sample Processing and Analysis of Paclitaxel

Each frozen plasma sample was added with 100 μl of dixipan as internal standard and 200 μl of 1.0 mol/L $NaHCO_3$. The mixture was added with 5 ml of diethyl ether, vortex-mixed, and centrifuged at 8000 rpm for 5 min to obtain a supernatant. The lower layer was added an equal volume of ether and operated as above, and the ether layers were mixed and dried with a nitrogen flow in 37°C water bath, and then the residue was reconstituted with 100 μl of methanol. Paclitaxel was detected by reversed-phase HPLC (Shimadzu System, Kyoto, Japan) (25).

Frozen tissues were homogenized with a certain volume of PBS into 15% or 25% homogenate with glass homogenizer. The homogenate was extracted, disposed as described above and analyzed by a modification of the HPLC method (26).

The HPLC method provided an assay that was both sensitive and specific for quantifying the paclitaxel. The recovery of paclitaxel from blood and various tissues was approximately 90% (data not shown).

Pharmacokinetic Study

Six female Japanese white rabbits (weighing 2.0–2.5 kg) were divided randomly into two groups of three rabbits each and were injected intravenously with paclitaxel magnetoliposomes or Cremophor EL/ethanol formulation at the paclitaxel dose of 3.5 mg/kg. Blood samples were taken from puncture of ear brim of rabbit into heparinized tubes at various

times (0, 0.017, 0.083, 0.25, 0.75, 2.5, 12, 24, 48, 72 h) and centrifuged at 8000 rpm for 10 min immediately. One milliliter of plasma sample was stored at −20°C until analysis (21).

In Vivo Distribution Studies

All female BALB/C mice were specific pathogen free animals (weighing 16–20 g) bred in the Laboratory Animal Center of Sichuan University. The EMT-6 breast cancer cell line (established from Institute of Cancer Research, Chinese Academy of Medical Sciences) has been shown to grow after 0.2 ml of 5×10^7 line cell per milliliter was injected subcutaneously into female BALB/C mice. The research protocols were approved in advance by the Institutional Animal Care and Use Committee of the University at Chengdu and conformed to the Principles of Laboratory Animal Care (27).

Viable EMT-6 breast tumor cells (1×10^6) with a volume of 0.2 ml were inoculated (day 0) subcutaneously into the area close to the right anterior armpit of female BALB/C mice (weighing 16–20 g). Thirty-six mice were randomly and equally divided into 12 groups; each 3 mice were injected intravenously at the paclitaxel dose of 10 mg/kg through caudal vein with paclitaxel in magnetoliposomes, conventional liposomes, or Cremophor EL/ethanol formulation, respectively, on day 9 after inoculation, when tumors had reached approximately 2 mm in diameter. The external magnetic field (the circular permanent magnetism tablet) was placed and fastened right on the skin surrounding the tumor mass by the sterilized rubberized fabric. The blood of three mice in each group was taken from the eye socket and the mice were sacrificed and dissected at designated time intervals (0.25, 2, 8, and 24 h) after administration of the preparation. The blood samples were placed in heparinized tubes, and then submitted to centrifugation at 8000 rpm for 10 min to obtain plasma samples. The upper plasma was frozen at −20°C until analysis. The tumor, heart, liver, spleen, lung, and kidney were isolated, weighed, and immediately frozen at −20°C until analysis.

On day 9, 36 mice in the other 12 groups were injected subcutaneously on the left chest wall with paclitaxel in above three modalities at the paclitaxel dose of 10 mg/kg and disposed by the same procedure described above. But the mice were sacrificed at the following sampling schedules: 2, 8, 24, and 48 h after subcutaneous dosing.

Antitumor Efficacy

Seventy-two female BALB/C mice (weighing 16–20 g) were divided randomly into 12 groups, each 6 mice, and were injected subcutaneously with EMT-6 breast tumor cells by the method described previously. Twenty-four hours later, different doses (20 mg/kg or 10 mg/kg) of modalities were given by subcutaneous administration on the left chest wall of the groups 1~4, intraperitoneally of the groups 7~10 of the infected mice once a day for 5 consecutive days, respectively. The external magnetic field (the circular permanent magnetism tablet) was placed and fastened right on the skin surrounding the tumor mass by the sterilized rubberized fabric.

Groups 5 and 11 were administered paclitaxel Cremophor-based formulation as the positive control, while groups 6 and 12 were administered physiologic saline as the negative control. On day 12 after inoculation, the mice of all groups were sacrificed and dissected, and the tumors were taken, weighed and compared.

The change rate of tumor weight was calculated using the equation given below:

$$\text{The change rate of tumor weight} = [(W_1 - W_2)/W_1] \times 100\%$$

Here, W_1 refers to the cancer weight of mice in the negative control group, and W_2 refers to the cancer weight of mice in the group cured.

Statistical Analysis

One-way ANOVA, Scheffe's F-test and Student's *t* test were used for statistical analysis to determine significant differences in paclitaxel concentrations among different experimental groups. Statistical significance was established at $p < 0.05$. Analyses were performed using the statistical package StatView, version 5.0 (SAS Institute, Inc., Cary, USA)

RESULTS AND DISCUSSION

Preparation of the Lyophilized Magnetoliposomes

It is the first time that the hydrophobic drug (paclitaxel) was entrapped into magnetoliposomes and the lyophilized formulation was evaluated fully in the decades. Several methods for preparing paclitaxel magnetoliposomes have been investigated in our laboratory, which include mechanic dispersing method (hand shaking method, ultrasonic method) and organic solvent dispersion method (ethanol injection method or ether injection method). The particle size of magnetoliposomes made by these methods was comparatively inconsistent. The formulations were unstable. Through a lot of preparatory experimentation and referring to the literature (9,27–29), the reverse evaporation method was chosen. Single factor tests were conducted to optimize the reaction conditions, including the amount of ethyl ether, the hydrogenated soybean phosphatidylcholine and cholesterol, the type and concentration of the buffer, temperature and time for vibration in the water bath, the mode of vibration, and so forth. Paclitaxel magnetoliposomes were then prepared according to the optimized conditions described above.

The prepared paclitaxel magnetoliposomes were suspension, which was unstable: its color became more intense and the drug precipitate was observed after being stored at room temperature (15~25°C) for 3 months. So freeze-drying or lyophilization of the formulation was performed to enhance the chemical and physical stabilities of the magnetoliposomes and minimize lipid hydrolysis. Glucose, lactose, and mannitol of different concentrations were used in screening tests of cryoprotectors for paclitaxel magnetoliposomes. Their redispersion ability was evaluated. After screening of the cryoprotectors, 8% lactose was chosen for freeze-drying because the lyophilized magnetoliposomes can redisperse easily in water. The lyophilized powder was hydrated with 0.02 mol/L of Tris-HCl buffer before use and was used in all the following tests if it was not pointed out particularly.

Paclitaxel is highly lipophilic and existed in the liposomal bilayers, and the ultrafine magnetite has high relative density and existed in the aqueous liposome core, so the existence of superfluous paclitaxel or ultrafine magnetite would destroy the liposomal structure and result in the accumulation and

precipitation of magnetoliposomes and the leakage of entrapped paclitaxel from magnetoliposomes. Although the addition of negatively charged lipid (DCP) will increase physical stability by preventing aggregation and fusion of magnetoliposomes due to electrostatic repulsion, the maximum amount of drug that could be loaded into the magnetoliposome bilayer was limited (about 4 mg), above this value, the formulation was not stable as the drug precipitated during liposome sizing or upon storage, and paclitaxel crystalline and liposome aggregation would be observed under the transmission electron microscope.

The inclusion of cholesterol in magnetoliposomes, even at low concentrations, generally inhibited to form the mixed micelle by restricting the flexibility of the hydrocarbon chain and reducing chain interaction and entanglement, and hence limiting the formation of phase separated lamellae (23,25).

Although most magnetoliposomes reported before were also prepared by reverse-phase evaporation method, the experimental conditions were apparently different from that of ours, such as the lipid molar ratio, the temperature, the sequence of adding materials in the process, and so forth (20,30). The difference was partly due to the different properties of the drugs [only hydrophilic drugs, such as diclofenac sodium (20), adriamycin (31,32), hydrazide pullula, and so forth (16) were entrapped into magnetoliposomes formulations before].

The lyophilized paclitaxel magnetoliposomes were homogeneous under naked eyes. And they would not cave in within 6 months. Under transmission electron microscope, they were small homogeneous vesicles and looked round and regular, easy to disperse. The mean diameter ($= \Sigma nd/\Sigma n$) was calculated to be 527 nm with 94.58% of the total being in the range of 200 nm~1002 nm. The span was 0.577 calculated by the following equation: $S = (D_{0.9} - D_{0.1})/2 \times D_{0.5}$. Here, S represents the span; $D_{0.9}$ and $D_{0.1}$ mean that 90% or 10% of the particles are smaller than the value given.

The sizes of magnetoliposomes were documented as 1.19 μm, 1.21 μm (20), 602.5 nm (33), 146 nm (31), 94.1 nm (34), 85.8 nm (32), and 80 nm (16) in former research separately, which were different from that made in the current study. The change in vesicle size may be due to different type, amount, particle size, and magnetic properties of dextran magnetite used before, the requirement of external magnetic field and the object of study. The vesicles were designed and prepared to achieve good magnetic responsiveness in our experiments. The result of the experiment *in vivo* confirmed our assumption.

Physical Properties and Stability

After dispersing in sterile water for injection, the suspension of the lyophilized paclitaxel magnetoliposomes showed pH value of 6.91 ± 0.01 (mean ± SD, n = 5). Electric properties were tested by electrophoresis in pH 7.4 PBS on cellulose acetate membrane at a voltage of 200 V. Under the experimental conditions, the paclitaxel magnetoliposomes were negatively charged. The relative viscosity of the paclitaxel magnetoliposome suspension was determined by an Ostward viscosity tube at 25 ± 0.5°C with sterile water for injection as the reference. The relative viscosity of the paclitaxel magnetoliposomes obtained was $1.0250 \times 10^{-3} \pm 6.3 \times 10^{-7}$ Pa s (mean ± SD, n = 5). The relative density of the paclitaxel magnetoliposome suspension was determined with a specific gravity bottle at 25 ± 0.5°C, using freshly boiled and cool water at the same temperature as the reference. The relative density of the paclitaxel magnetoliposomes suspension was obtained as $1.1105 \pm 1.92 \times 10^{-4}$ (n = 5) (mean ± SD, water as 1).

Previous work has shown that cationic liposomes may be transported to the brain, while negatively charged liposomes could be optimal for cellular delivery (23,35,36). Therefore, we designed and prepared the negatively charged liposomes by adding the negatively charged dicetylphosphate to the phospolipidic bilayer.

All lyophilized liposomes were sealed in vials and stored at 3~5°C, 15~25°C, and 37°C/75% relative humidity for 3 months, respectively. The stability parameters, such as particle size distribution and drug entrapment efficiency, were determined as a function of the storage time. After storage for 3 months at 3~5°C, 15~25°C, and 37°C/RH75% (relative humidity), lyophilized conventional or magnetoliposomes containing paclitaxel showed almost no change in appearance, re-dispersing ability, particle size, and entrapment efficiency. No drug precipitation or liposomal aggregation was observed during storage. These results indicated that lyophilized paclitaxel magnetoliposomes were physically stable under the storage conditions. In our study, cholesterol, which has a bilayer-rigidifying effect, has a positive effect, probably because it contributes to making the magnetoliposomes more resistant to the exchange of phospholipids with serum high-density lipoproteins.

Quantification of Paclitaxel and Determination of Entrapment Efficiency

The separated paclitaxel was analyzed by reversed-phase HPLC (Shimadzu System). The drug concentration was calculated from standard curves. The standard regression equation in the range of C = 0.803~8.026 μg/ml was linear ($Y = -0.00059 + 0.19452C$, $r = 0.9994$; Y means the peak area ratio between paclitaxel and dixipan) (21). The recoveries of paclitaxel are 98.22% ± 0.48% (mean ± SD, n = 5), which showed that the above method was reliable for determination under the described conditions. The percentage of paclitaxel into magnetoliposomes was found to be 97.25% ± 0.92% (mean ± SD of 5 trials). The high drug entrapment efficiency further confirmed the high association of drug with magnetoliposomes.

Paclitaxel belongs to diterpene compound, which is highly lipophilic and insoluble in water, so paclitaxel liposomes were usually separated from free paclitaxel following the procedures described below, in brief, the first method: paclitaxel liposomes were diluted by PBS, centrifuged, the precipitation was taken out, dried at vacuum, added with methanol, and the free paclitaxel existing in the precipitate was measured. The second method: paclitaxel liposomes were passed through Sephadex G mini-column to remove unentrapped drug. The liposomes were lysed using minimum amount of Triton X-100 (0.5% w/v), and drug content was determined by the spectrophotometer. But in the first method, paclitaxel magnetoliposomes precipitated with the free drug together due to existence of the ultrafine magnetite, and in the second method, it took a long time to separate the magnetoliposomes from the free drug and the result was not

very satisfactory (not reported). So, we chose another method described above to measure the free paclitaxel and then the entrapment efficiency was calculated.

Pharmacokinetic Study

Paclitaxel plasma concentration was determined and calculated according to the method described above, as shown in Table I.

The models were described in one-compartment, two-compartment, and three-compartment separately, and were optimized in terms of F-test, the goodness of fit, Akaike's Information Criteria (AIC), and r^2; the results were obtained as follows: both of the pharmacokinetic behaviors of paclitaxel magnetoliposomes and paclitaxel Cremophor EL/ethanol formulation confirmed to three-compartment model with intravenous dosage. The pharmacokinetic equations were described as follows, respectively:

$$C = 24.9098e^{-21.7405t} + 3.6659e^{-1.3507t} + 0.2164e^{-0.0358t}$$

$$C = 16.1884e^{-26.7302t} + 4.9018e^{-1.6384t} + 0.4875e^{-0.1687t}$$

Table II shows the pharmacokinetic parameters of different modalities after i.v. administration into the rabbits. The entrapment of paclitaxel into magnetoliposomes altered disposition of the paclitaxel *in vivo*, which produced marked differences in terms of the pharmacokinetic parameters. In particular, the values of $t_{1/2\beta}$ and the area under the concentration-versus-time curve (AUC) were found to be much higher for paclitaxel vectored by magnetoliposomes than for paclitaxel in Cremophor EL/ethanol. Paclitaxel in magnetoliposomes were relative long circulating ($t_{1/2\beta}$ 19.37 h) compared to the drug in Cremophor EL/ethanol ($t_{1/2\beta}$ 4.11 h); the AUC of paclitaxel magnetoliposomes were 3-fold higher than that of the latter. Furthermore, the clearance parameters of magnetoliposomes and Cremophor-based formulation were 0.5047 and 0.7706 L kg/h, respectively, which showed that the clearance rate of both dosages from the rabbit plasma were slow and the former was slower than the latter.

In Vivo Distribution Studies

To evaluate *in vivo* uptake of paclitaxel by tumor, heart, liver, spleen, lung, and kidney, three modalities, including paclitaxel in Cremophor EL/ethanol, conventional and magnetic liposomes, were respectively injected and the drug concentration in mouse tissue was determined after organ digestion.

Table I. Paclitaxel Plasma Concentration in Different Modalities After i.v. Administration

Time (h)	Paclitaxel concentration (μg/ml, mean ± SD, n = 3)			
	Magnetoliposomes		Cremophor EL/ethanol	
	1	2	1	2
0.017	20.71 ± 0.55	21.01	15.53 ± 0.57	15.53
0.083	7.84 ± 0.43	7.59	6.52 ± 0.28	6.52
0.25	2.65 ± 0.06	2.94	3.74 ± 0.1	3.74
0.75	1.91 ± 0.08	1.54	1.87 ± 0.1	1.86
2	0.42 ± 0.04	0.45	0.52 ± 0.02	0.53
5	0.19 ± 0.01	0.19	0.23 ± 0.03	0.21
12	0.14 ± 0.05	0.14	0.05 ± 0.01	0.06
24	0.57 ± 0.04	0.09	ND	0.01
48	0.24 ± 0.05	0.04	ND	0
72	ND	0	ND	0

1, experimental concentration; 2, theoretical concentration (Co); ND, not determined because the amount was under the determination limitation.

Table II. Pharmacokinetic Parameters of Paclitaxel Modalities After i.v. Administration Into Rabbits

Parameter	Unit	Values	
		Magnetoliposomes	Cremophor EL/ethanol
$t_{1/2pi}$	h	0.0319	0.0259
$t_{1/2\alpha}$	h	0.5132	0.4231
$t_{1/2\beta}$	h	19.3706	4.1099
K_{12}	1/h	11.9544	15.1757
K_{21}	1/h	4.0565	7.8234
K_{13}	1/h	4.1205	1.9286
K_{31}	1/h	0.0891	0.2839
K_{10}	1/h	2.9064	3.3257
AUC	h μg/ml	20.7153	6.7958
Cl(s)	L/h kg	0.5047	0.7706

After i.v. bolus administration, in tumor, average paclitaxel concentration with magnetoliposomes were much higher than that with conventional liposomes and Cremophor EL/ethanol all the time, and the drug concentration with the former modality decreased much slower than with the latter modality, as shown in Fig. 1. Compared with Cremophor EL/ethanol and conventional liposomes, the paclitaxel level in tumor with magnetoliposomes was approximately 29.82 and 4.06 times higher at 24 h, 4.93 and 2.61 times higher at 8 h, 1.10 and 1.31 times higher at 2 h, and 1.25 and 1.83 times higher at 0.25 h. Furthermore, in the case of magnetoliposomes, the paclitaxel concentration in the tumor was much higher than that in plasma, heart, liver, spleen, lung, and kidney tissues, especially for the extended time (4.88, 42.65, 1.99, 1.38, 1.66, and 7.87 times higher at 8 h; 50.7, 15.84, 2.37, 0.59, 1.98, and 5.45 times higher at 24 h, respectively). *In vivo* distribution studies explicitly suggested that magnetoliposomes were distinctively localized in the tumor tissues. Moreover, under the influence of external magnetic field, the magnetoliposomes seemed to reach the concentration peak soon (19.85 μg/g at 0.25 h) and remained almost the same level at the site of magnetic field application up to 2 h, then decreased slowly in the tumor over time due to the drug clearance half-life and metabolism. The results strongly suggested that paclitaxel magnetoliposomes had obvious tumor-targeting and sustained-release effect due to selective uptake of magnetoliposomes, which have good responsiveness to magnetic field.

Sanyog *et al.* (20) reported that in case of negatively charged magnetic liposomes, drug level in the target site (brain) was 5.95-fold compared to free drug and 7.58-fold in comparison to nonmagnetic formulation after 4 h. Liver uptake was significantly bypassed for magnetic liposomes (relative reduction in accumulation level was 37.2%). Gupta *et al.* (37) compared the comparative disposition of adriamycin delivered via magnetic albumin microspheres in presence and absence of magnetic field in rats. Administration of magnetic doxorubicin liposomes following application of a 0.4 T magnetic field produced a 3- to 4-fold higher maximum doxorubin concentration in the tumor (32). Selective accumulation of magnetite particles suggested that this systemic chemotherapy could effectively control the primary tumor without

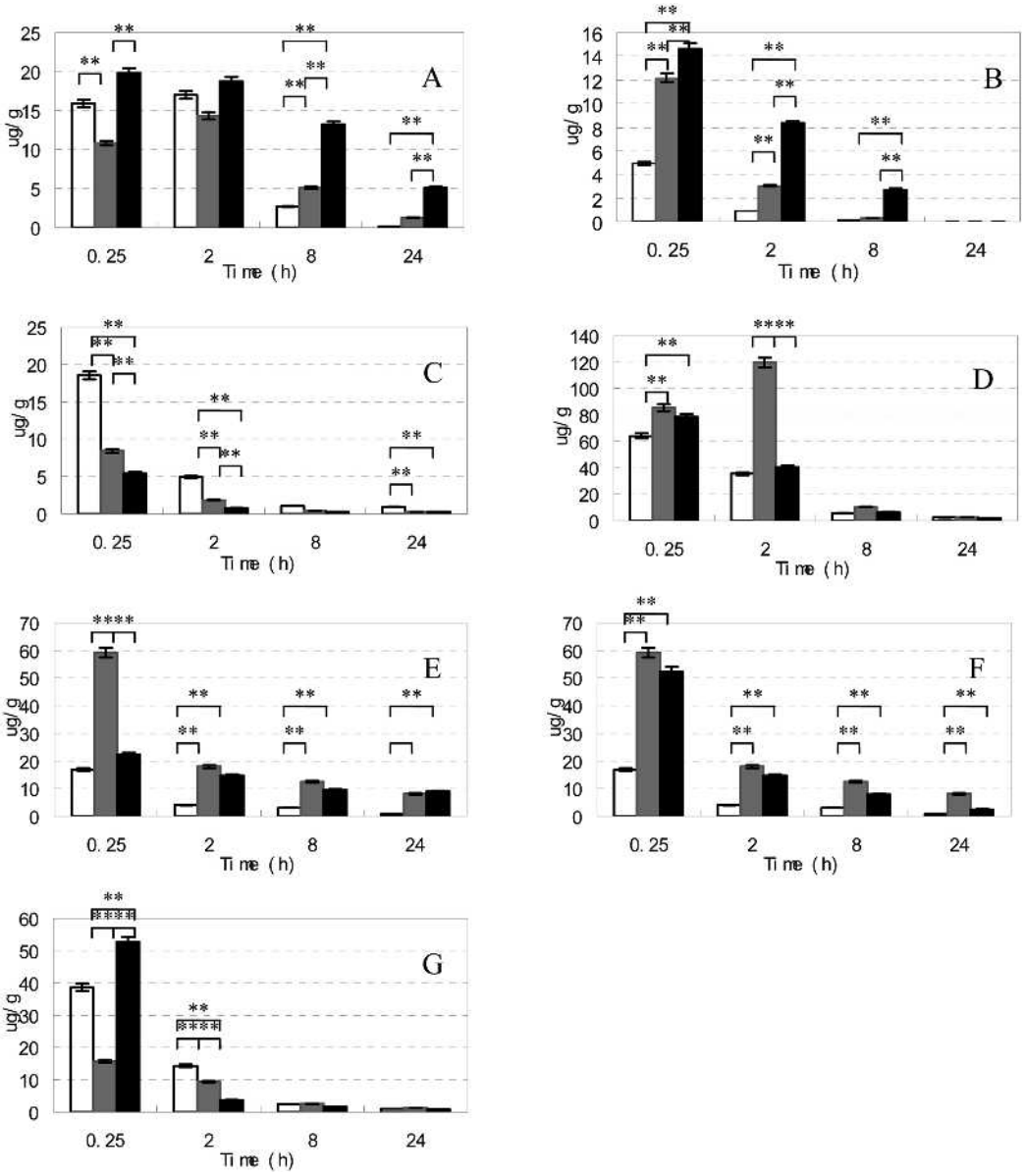

Fig. 1. *In vivo* distribution in BALB/C mice of paclitaxel Cremophor EL/ethanol-based formulation (□), conventional (▨), and magnetoliposomes (■) in tumor (A), plasma (B), heart (C), liver (D), spleen (E), lung (F), and kidney (G) after intravenous administration. Standard deviations (not reported) were below 5% of the mean values. Histograms represent the arithmetic means of three determinations (n = 3 animals per group). Paclitaxel concentration was determined by reverse-phase HPLC. **p < 0.01 showed significantly different between groups as evaluated by Scheffe's F-test.

significant side effects, due to the targeting of magnetic liposomes. In other words, magnetic carriers were restricted in the tumor due to good magnetic responsiveness. Such targeted drug delivery for anticancer agents would provide clinical advantages compared to current methods.

Only paclitaxel with magnetoliposomes was still remaining high concentration (2.71 μg/ml) in the plasma up to 8 h compared with conventional liposomes (0.33 μg/ml) or Cremophor EL/ethanol (0.15 μg/ml). The plasma clearance rate of paclitaxel in magnetoliposomes was the lowest compared with those of conventional liposomes and Cremophor EL/ethanol. Liposomal formulation containing a small fraction of ultrafine magnetite has appeared to alter dramatically the pharmacokinetic properties of paclitaxel, leading to long distribution half-life and small volume of distribution. These results clearly confirmed again that magnetoliposomes could be thought of as depositories and contributed to the sustained effect. These results also revealed that the release of paclitaxel from magnetoliposomes was slow and continuous in contrast to the fast release from conventional liposomes or Cremophor EL/ethanol. Paclitaxel-loaded magnetic carriers can be guided to and retained at the target site (tumor) with the help of an external magnetic field of appropriate strength. In turn, retention of magnetic liposomes at target site apparently delayed plasma clearance and neodisposition of the contained drug.

To evaluate the *in vivo* uptake circulation by cells of the mononuclear phagocytic system (MPS), liver and spleen dis-

tributions of conventional and magnetic liposomes containing paclitaxel were examined. For conventional liposomes, paclitaxel was rapidly taken up to an extent by the liver and spleen, followed by a progressive and slow decrease of drug concentration in these organs, except the concentration in liver was up to maximum at 2 h. Paclitaxel concentration from conventional liposomes was much higher than that from magnetoliposomes at any time points. Biodistribution study in mice showed a considerable decrease in drug uptake in mononuclear phagocytic system (MPS)-containing organs (liver and spleen) with magnetoliposomes as compared to conventional liposomes. Moreover, through modifying liposome membrane, the quantity and rate of uptake by reticuloendothelial system would decrease less, liver and spleen uptake was avoided to a significant extent, and the clearance rate in plasma would simultaneously debase much.

Paclitaxel concentrations of magnetoliposomes in heart were much lower than those of both conventional liposome and Cremophor/ethanol-based formulation. This result indicated that magnetoliposomes benefited to increase the anticancer efficiency and decrease the toxicity and side effects.

Hence, *in vivo* organ distribution studies showed that paclitaxel concentration with magnetoliposomes remained much high level in tumor or plasma under the influence of external magnetic field, while conventional liposomes were mainly taken up by organs of RES; therefore, much less concentration was found in tumor and plasma.

After s.c. bolus administration, the drug level of magnetoliposomes in tumor showed much higher than those of conventional liposomes and Cremophor EL/ethanol-based formulation within 48 h, followed by the slowest decrease of drug concentration, as shown in Fig. 2. This suggested that paclitaxel magnetoliposomes had obvious tumor-targeting and sustained-release effect, while drug concentration in heart, liver, spleen, and kidney were much lower than those of both conventional liposome and Cremophor EL/ethanol-based formulation. These results indicated that magnetoliposomes benefited to increase the anticancer efficiency and lessen the toxicity and side effects.

Hence, our studies showed that these two different administration routes could result in similar drug distribution *in vivo*. In general, the recorded values of paclitaxel concentration in the case of subcutaneous administration are much less than those of intravenous administration. This may be attributed to the time delay during the drug transportation process to the circulation in subcutaneous administration.

In former studies (19,31), a permanent magnet of 0.4 T was implanted in the center of tumor mass; however, use of an external magnetic force is clinically more convenient than surgical implantation of a magnet because the application time and site can be changed according to the clinical demands. Based on these considerations, in the current study we evaluated the efficiency of systemic chemotherapy with small magnetic paclitaxel liposomes in EMT-6 sarcoma-bearing mice, using a circular permanent magnet tablet (0.2 T) as an external magnetic force. The external magnetic field (the circular permanent magnet tablet) was placed and fastened right on the skin surrounding the tumor mass by the sterilized rubberized fabric. Paclitaxel magnetic liposomes exhibited good magnetic responsiveness to the external magnetic field *in vivo.* There was no lesion to the mice with this method compared to the surgical method. And the activities of all mice were not affected by the external permanent magnet during the whole experimental period.

Antitumor Efficacy of Paclitaxel Magnetoliposomes

Previously, Shinkai *et al.* (16) found that when magnetoliposomes were incubated with cancer cells, they were absorbed onto the cell surface and incorporated by the cells about 12 times more effectively than the control after 4 h. This result elucidated clearly that magnetoliposomes enable concentrate selectively the target cells and have high affinity for tumor cells (38). The apoptotic index in group with magnetoliposomes injection and hyperthermia was 22.9%, significantly higher than in group control (34), which indicated that magnetoliposomes injected into the tongue could target cervical lymph node metastases and accumulate there at concentrations sufficient to generate therapeutically effective temperatures. The magnetic liposomes developed by Hiroo *et al.* (32) showed that newly designed magnetic systemic chemotherapy significantly suppressed primary tumor growth for at least 2 weeks and effectively reduced lung metastasis at 3 weeks posttreatment in hamsters due to increased targeting of doxorubicin. The studies conducted by Alexios *et al.* (39) or Kubo *et al.* (31) also confirmed the effectiveness of such magnetic treatment modalities. Lubbe *et al.* (40) already achieved complete tumor remission in animals using a new kind of ferrofluid associated with epirubicin and external magnetic field at 0.5–0.8 T. In a second step by the same authors (41), the phase I clinical trial using this approach was performed on patients with advanced, unsuccessfully treated cancers or sarcomas with very promising results. In all, the effective antitumor efficacy to cure some tumors was well recognized via intravenous administration adopted in all of above studies. Based on consideration of the need of extensive clinical use of paclitaxel to cure breast cancer, the antitumor efficacy of paclitaxel magnetoliposomes via two common administrations currently in clinical use (s.c. and i.p.) were evaluated here.

In our investigation, Table III shows that the change rates of tumor weight after subcutaneous (s.c.) administration were between 46.5~60.5%. When the tumor was treated by paclitaxel magnetoliposomes, the rate was the highest (60.5%) compared with that by paclitaxel in Cremophor EL/ethanol-based formulation or conventional liposomes of the same dose. Given at half dose (10 mg/kg), paclitaxel in magnetoliposomes was nearly equally potent (50.4%) to paclitaxel in Cremophor EL/ethanol (51.9%) or in conventional liposomes (53.5%). The antitumor efficacy of active drug delivery system (magnetoliposomes) with an external magnetic force exceeded that of paclitaxel in conventional liposomes or in Cremophor EL/ethanol treatments because of the enhanced delivery of magnetoliposomal paclitaxel to tumors and the retention of the magnetic liposomes at the target site. Moreover, the antitumor effect was positively correlated with the dose administration, while the toxicity of paclitaxel magnetoliposomes were very low (described in a later section), so it was possible to administer subcutaneously more paclitaxel magnetoliposomes in order to achieve much better antitumor effect in further studies. We believe that the better antitumor efficacy shown by the magnetic paclitaxel formulation compared to conventional liposomes might be due to the site local effect under the guidance of an externally applied magnetic field, which increased the time required for tumor cell uptake

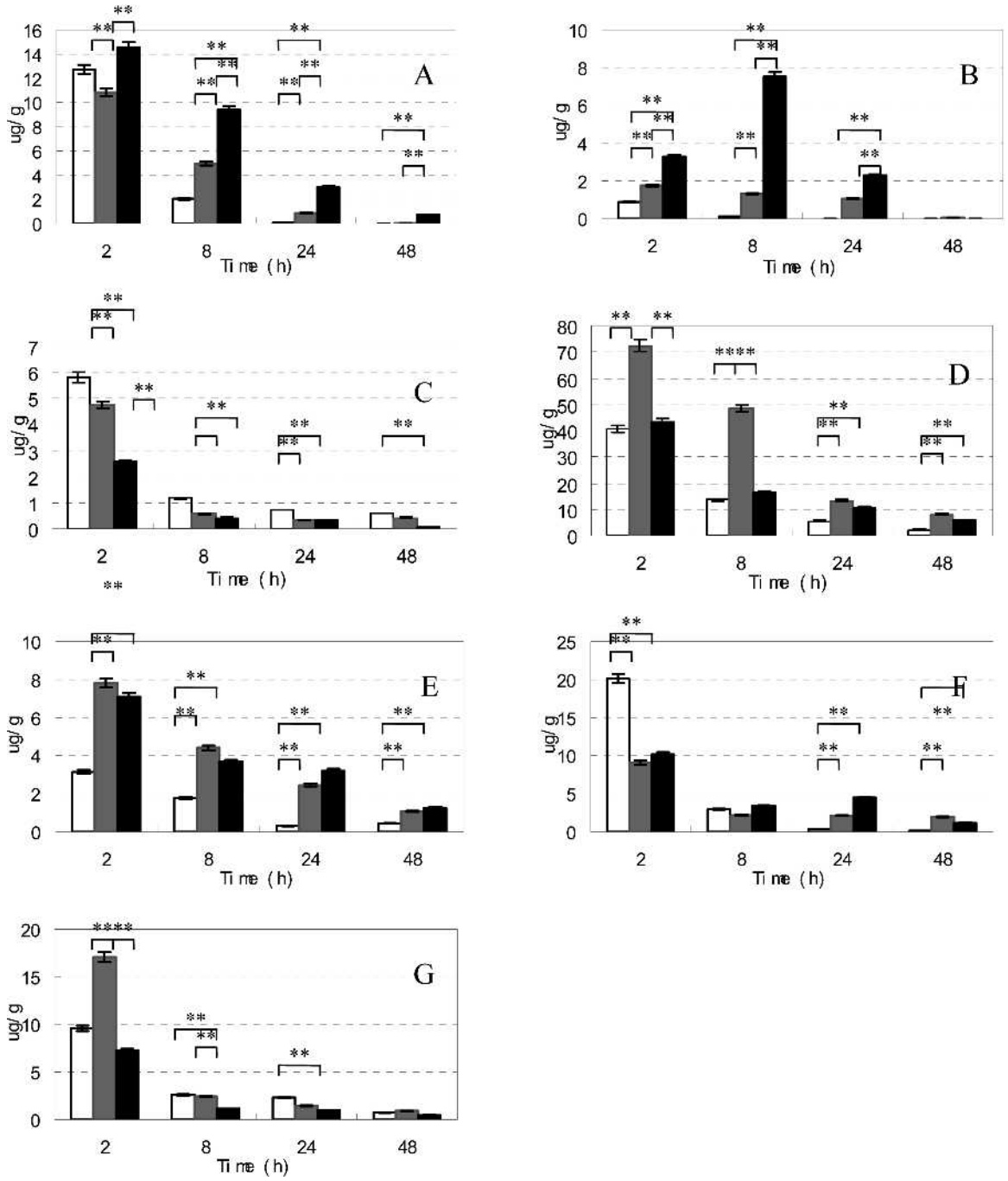

Fig. 2. *In vivo* distribution in BALB/C mice of paclitaxel Cremophor EL/ethanol-based formulation (□), conventional (▨), and magnetoliposomes (■) in tumor (A), plasma (B), heart (C), liver (D), spleen (E), lung (F), and kidney (G) after subcutaneous administration. Standard deviations (not reported) were below 5% of the mean values. Histograms represent the arithmetic means of three determinations (n = 3 animals per group). Paclitaxel concentration was determined by reverse-phase HPLC. **$p < 0.01$ showed significantly different between groups as evaluated by Scheffe's F-test.

and drug internalization. In other words, the ability to control and produce efficient release would be extremely advantage as wanted in order to provide therapeutic benefit.

In similar manner, Table IV shows that the antitumor rates after intraperitoneal administration were between 41.1~51.7%. As expected, when the tumor was treated by paclitaxel magnetoliposomes, the rate was the highest (51.7%) compared with those by the other two paclitaxel formulations. It was also strongly suggested to intraperitoneally administer more paclitaxel magnetoliposomes to achieve much better antitumor effect in further studies.

When antitumor drug of the same low dose (10 mg/kg) was given, both change rates of tumor weight (50.4% in s.c., 43.7% in i.p.) demonstrated a very significant difference ($p < 0.01$) in control. However, the better antitumor efficacy achieved by s.c. administration may be due to the shorter

Table III. Antitumor Effect of Paclitaxel Modalities When They Were Administered Into Mice Bearing Breast Carcinoma by s.c. Administration (n = 6)

Drug	Group no.	Dose (mg/kg)	Weight of tumor (g, mean ± SD)	Change rate of tumor weight (%)
1	1	20	0.51 ± 0.11	60.5**
	2	10	0.64 ± 0.27	50.4**
2	3	20	0.60 ± 0.13	53.5**
	4	10	0.69 ± 0.19	46.5**
3	5	20	0.62 ± 0.19	51.9**
4	6	0	1.29 ± 0.42	

1, paclitaxel magnetoliposomes; 2, paclitaxel conventional liposomes; 3, paclitaxel in Cremophor EL/ethanol formulation as positive control; 4, physiological saline as negative control.
** $p < 0.01$, significantly different as evaluated by Scheff's F-test.

distance between the drug injection site and the tumor site compared with that by i.p. administration. The highest change rate of tumor weight (60.5%) was achieved by s.c. administration partly because the different maximum dose (20 mg/kg by s.c. or 15 mg/kg by i.p., respectively) given and the aforementioned distance between the tumor and the injection site.

In short, the antitumor potency of the paclitaxel magnetoliposomes was superior to that of Cremophor EL/ethanol-based formulation or conventional liposomes. The paclitaxel magnetoliposomes showed a significant increase in antitumor potency.

The Results of Stimulation and Erythrocatalysis Test of Paclitaxel Magnetic Preparation

One milliliter of paclitaxel conventional or magenetic liposomes was administered into one side of the thigh quadriceps of two rabbits' hind limbs, respectively. Thigh quadriceps of the two other sides of the hind limbs were injected with penicillin G or physiologic saline at the same volume as positive or negative control. Forty-eight hours later, the rabbits were sacrificed by letting blood, and the thigh quadriceps were taken out and cut vertically. The changes of the muscles were observed and the reaction extent was divided into six grades. The diameter of the hyperemia area in the muscles of rabbits after injection of paclitaxel magnetoliposomes was about 0.5 cm. The results showed that the paclitaxel magnetoliposomes were suitable for intramuscular or subcutaneous administration.

Two percent red cell suspension was prepared from fresh rabbit blood. The conventional or paclitaxel magnetoliposomes of different volumes were added into the suspension to a total volume of 2.5 ml. The mixture in tubes was put into 37°C water bath, and then the effect of the liposomes on red cell was observed at 0.25 h, 0.5 h, 1 h, 2 h, and 3 h. The suspension without any liposomes was used as the negative control and the suspension with distilled water as the positive control. There were no erythrocatalysis and agglomeration phenomenon observed for paclitaxel magnetoliposomes, which showed that this paclitaxel modality was suitable for subcutaneous or intravenous administration.

Table IV. Antitumor Effect of Paclitaxel Modalities When They Were Administered Into Mice Bearing Breast Carcinoma by i.p. Administration (n = 6)

Drug	Group no.	Dose (mg/kg)	Weight of tumor (g, mean ± SD)	Change rate of tumor weight (%)
1	7	15	0.73 ± 0.13	51.7**
	8	10	0.85 ± 0.18	43.7**
2	9	15	0.83 ± 0.25	45.0*
	10	10	0.89 ± 0.21	41.1*
3	11	10	0.85 ± 0.27	43.7*
4	12	0	1.51 ± 0.47	

1, paclitaxel magnetoliposomes; 2, paclitaxel conventional liposomes; 3, paclitaxel in Cremophor EL/ethanol formulation as positive control; 4, physiological saline as negative control.
* $p < 0.05$; ** $p < 0.01$, significantly different as evaluated by Scheff's F-test.

Acute Toxicity

The preclinical toxicology of paclitaxel has been explored on 100 kunming mice weighing 18~22 g in this study. The subcutaneous and interperitoneal routes of administration were used because of dose-volume constraints that were imposed by paclitaxel's limited aqueous solubility and the toxicity of its Cremophor EL/ethanol vehicle. For paclitaxel Cremophor EL/ethanol formulation, LD_{50} (with 95% confidence limit) for subcutaneous or intraperitoneal administration was calculated to be 147.3 (125.83~159.66) mg/kg and 53.30 (43.09~65.92) mg/kg (data not reported). Everyday activities of the mice receiving s.c. injection decreased, and some mice scratched the injection area with the halluxes and canker. The mice by intraperitoneal injection twisted the bodies. The dead mice were dissected, and inflating and dilatation of stomach and intestines was found. In the new drug application material to the Chinese State Food and Drug Administration provided by the Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences (P.R. China), LD_{50} for subcutaneous or intraperitoneal administration was reported to be 152.17 (124.42~186.11) mg/kg and 61.52 (50.99~74.22) mg/kg, respectively. However, LD_{50} was reported to be 206 mg/m^2 (about 86 mg/kg) when the mice were intraperitoneally administered once a day for 5 days (42).

LD_{50} of conventional or paclitaxel magnetoliposomes could not be determined by single injection due to the concentration and volume limitation. Therefore, the maximum tolerated dose (MTD) for paclitaxel-liposome formulations was determined by subcutaneous or intraperitoneal administration in healthy mice in our study. MTD experiments were performed on 20 animals in one group. Paclitaxel magnetoliposomes were dispersed to their maximum concentrations and the acceptable maximum volumes for single time were injected into the mice. Briefly, 30 or 42.86 mg of the lyophilized liposomal powder was taken from 7 or 10 vials (containing 4.286 mg per vial) for magnetoliposomes or conventional liposomes, repectively, and reconstituted in 10 ml of 0.02 mol/L of Tris-HCl buffer. Then the dose of 300 mg/kg was given into 20 mice subcutaneously or 428.6 mg/kg was given intraperitoneally, and the death rates recorded on day 7 after administration were 0% and 10%, respectively. When conventional liposomes of the paclitaxel dose 428.6 mg/kg were injected, the death rates were 0% and 35%, respectively (data not reported).

Everyday activities of the mice decreased on the day that paclitaxel magnetic and conventional liposomes were given, but reconverted on the following day. The mice usually died from day 4 to day 7. The dead mice were dissected, and no obvious changes were observed in the organs such as heart, liver, spleen, lung, and kidney. The surviving mice remained normal up to day 7.

It was obvious that the acute toxicity of both paclitaxel magnetic and conventional liposomes were much lower than that of paclitaxel formulated in Cremophor EL/ethanol. Meanwhile, systemic toxicity and side effects produced by the usage of Cremophor EL/ethanol could be avoided entirely through entrapping paclitaxel into magnetic and conventional liposomes.

CONCLUSIONS

The current study investigates magnetic liposomes designed to act as anticancer drug carriers, which can be effectively delivered to solid tumors via parental administration. The lyophilized and negatively charged paclitaxel magnetoliposomes developed in our study were shown to be selectively taken up by the tumor and actively targeted to some poorly accessible site (i.e., breast cancer), thus avoiding the uptake by fixed macrophages of the liver and spleen, because of their exclusive migration tendency toward tumor under the guidance of external magnetic field. Magnetoliposomes could effectively and preferentially delivery paclitaxel to the tumor after parenteral administration. Therefore, the antitumor efficiency of the paclitaxel magnetoliposomes is superior to that of the paclitaxel Cremophor EL/ethanol formulation or paclitaxel conventional liposomes. And the paclitaxel magnetoliposomes could increase significantly the antitumor efficiency and sustained effect of paclitaxel, meanwhile lessen the side effects and acute toxicity. This study opens a new perspective of active delivery of hydrophobic drugs for a possible treatment of breast carcinoma via parenteral administration. Furthermore, the possibility of paclitaxel targeting using a static magnetic field was provided here with very promising results, and this method would be helpful in treating a diseased organ by first targeting magnetoliposomes and subsequently exposing to the external magnet field. Magnetic targeting may have wide applications because it is not organ-specific (34,43), therefore, represents a novel versatile tool for cancer treatment. This targeted drug delivery system for anticancer agents could provide numerous clinical advantages over existing systems.

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