European Review for Medical and Pharmacological Sciences 2013; 17: 3347-3361

PEG-coated irinotecan cationic liposomes improve the therapeutic efficacy of breast cancer in animals

L. ZHANG, D.-Y. CAO, J. WANG, B. XIANG, J.-N. DUN, Y. FANG, G.-Q. XUE

Department of Pharmaceutics, School of Pharmaceutical Sciences, Hebei Medical University, Research Center of Chinese Medicine Injection in Hebei Province, Shijiazhuang, China

Abstract. – BACKGROUND: Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females owing.

AIM: This study aimed to construct a kind of PEG-coated irinotecan cationic liposomes for investigating its efficacy and mechanism of action in the treatment of breast cancer in preclinical models.

MATERIALS AND METHODS: Evaluations were performed on the MDA-MB231 breast cancer cells, the xenografted MDA-MB231 cancer cells in Female nude mice and Sprague-Dawley (SD) rat. The liposomes were characterized through assays of cytotoxicity, intracellular uptake, nuclei morphology, antitumor activities, pharmacokinetics and tissue distribution.

RESULTS: The zeta potential of PEG-coated irinotecan cationic liposomes was approximately 23 mV. The PEG-coated irinotecan cationic liposomes were approximately 66nm in diameter, significantly increased the intracellular uptake of irinotecan, and showed strong inhibitory effect on MDA-MB231 breast cancer cells. A significant antitumor efficacy in the xenografted MDA-MB231 breast cancer cells in nude mice was evidenced by intravenous administration of PEG-coated irinotecan cationic liposomes. PEG-coated irinotecan cationic liposomes also improved the irinotecan blood circulation time and showed an enhanced drug concentration in tumor.

CONCLUSIONS: PEG-coated irinotecan cationic liposomes had significant inhibitory effect against breast cancer *in vitro* and *in vivo*, hence providing a new strategy for treating breast cancer.

Key Words:

Breast cancer, PEG-coated irinotecan cationic liposomes, Pharmacokinetics, Tissue distribution, HPLC.

Introduction

Breast cancer is one of the most frequent cancers among women worldwide. It is a disease that can affect women of various ages where the risk of developing breast cancer increases with age[1,2]. Approximately one in eight women will be diagnosed with breast cancer during their lifetime. As a consequence, there is a great need to develop a treatment strategy that can successfully reduce the incidence of breast cancer and prolong the life of affected women[3,4].

Irinotecan (CPT-11), a water-soluble camptothecin, is commonly in clinical used for the cancer treatment. It exerts anti-tumor activity by inhibiting the intranuclear enzyme topoisomerase I[5,6]. In tumor cells, the level of topoisomerase I enzyme is higher than in normal cells. Irinotecan was shown to exert its cytotoxic activity through inhibition of DNA replication while acting upon DNA topoisomerase I enzyme[7,8]. DNA topoisomerase relieves the torsional stress that develops during DNA replication by inducing single strand breaks. The irinotecan binds to the topoisomerase I-DNA complex, thereby leading to replication arrest and the formation of double-strand DNA breaks which, while not repaired, can lead to the cancer cell death. However, the main adverse effects of irinotecan in humans are gastrointestinal toxicity and myelosuppression which limits its usage and administration[9,10].

Liposomes have been widely used in the therapeutic drug delivery to enhance permeability and retention (EPR) effects in tumor tissues and reduce systemic side effect of anticancer agent, including small molecular drugs, proteins, genes (DNA or RNA) and diagnostic contrast reagents[11,12]. PEGylated lipids (PEG_{2000}-DSPE) can inhibit opsonization by plasma proteins and contribute longer circulation for liposome because of the "steric stabilization" effect. With the surface hydrophilic protective layer from PEG chain, PEGylated liposome showed characterizations of more stability, sustained release, prolonged blood circulation time and reduced reticuloendothelial system uptake[13,14].

Although liposomes have been successfully applied in the clinic, further efforts on the component optimization are still a hot topic in the re-

Corresponding Author: De-Ying Cao, Ph.D; e-mail: cao_dy@126.com

search for satisfying requirements from clinic. A change of the liposomes' charge is believed to be one of the key factors affecting cellular adhesion/uptake and drug delivery[15]. Liposomes with cationic charge are prone to binding cells than liposomes with neutral or anionic lipids due to electrostatic interaction with negatively charged molecule on tumor cell membrane (glycoproteins, anionic phospholipids and proteoglycans). This is the reason that cationic liposomes were frequently used to improve *in vitro* and *in vivo* efficacy for drug delivery[16,17].

The objectives of the present study are to prepare polyethylene glycol (PEG)-coated irinotecan cationic liposomes by incorporating octadecylamine into the lipophilic bilayer, as shown in Figure 1A. Irinotecan was entrapped into the aqueous cores by ammonium sulfate gradient method in order to get high encapsulation efficiency[18]. The aim of present study was to explore the preparation of PEG-coated irinotecan cationic liposomes, to define the action mechanisms, to evaluate the efficacy in treating of breast cancer, and to evaluate the pharmacokinetics and distrib-

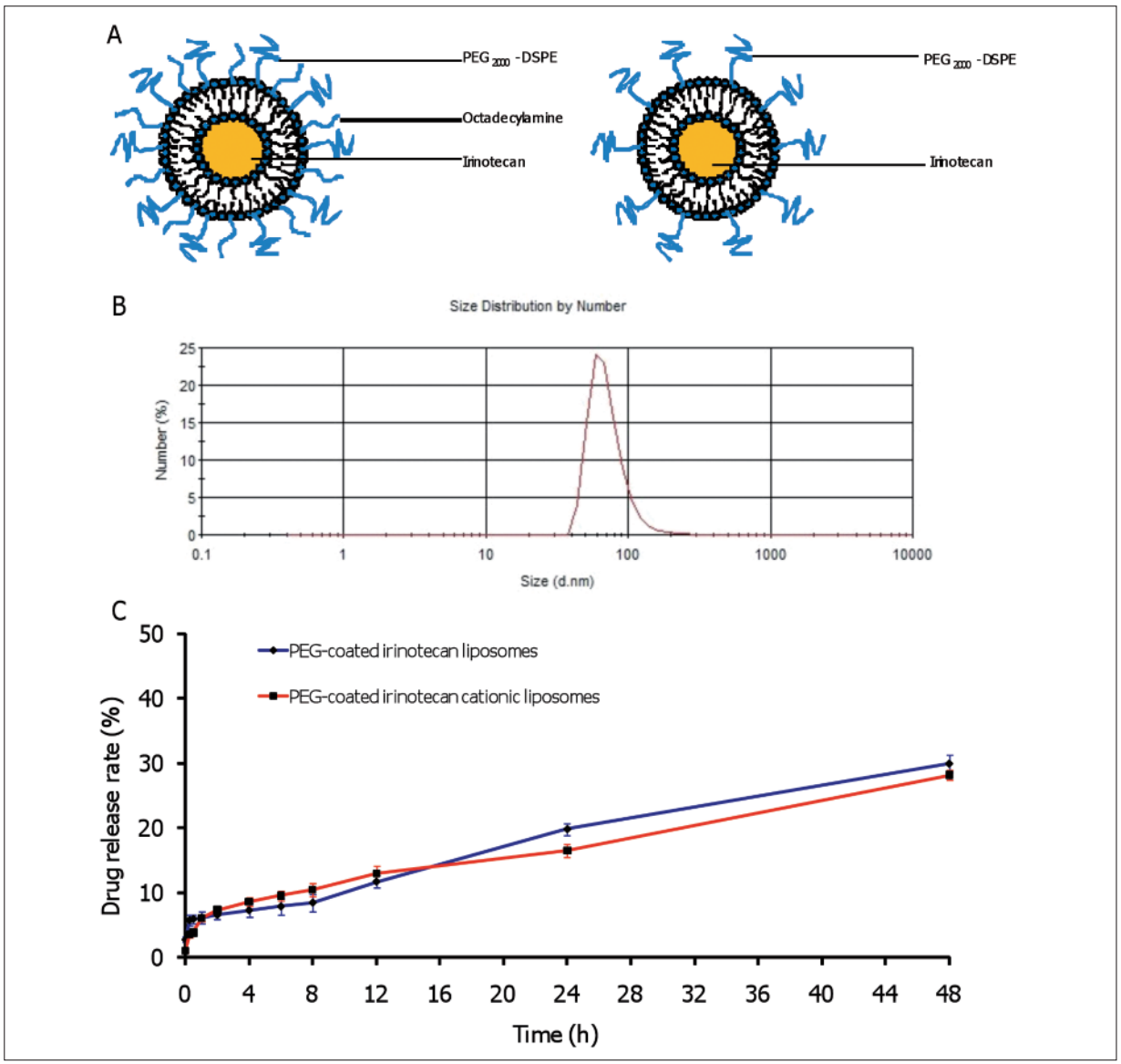


Figure 1. ***A,*** Schematic representations of two liposomal constructs consisting of PEG-coated irinotecan cationic liposomes and PEG-coated irinotecan liposomes. ***B,*** Size distribution of PEG-coated irinotecan cationic liposomes by dynamic light scattering (DLS) analysis. ***C,*** The release profiles of irinotecan from two different liposomes in PBS solution containing 10% serum protein at 37°C respectively. Data are presented as the mean ± SD (n=3).

ution of irinotecan after intravenous administration of PEG-coated irinotecan cationic liposomes.

Materials and Methods

Preparation of PEG-Coated Irinotecan Cationic Liposomes

PEG-coated irinotecan cationic liposomes were prepared as previously described. Soybean phosphatidylcholine (SPC) (Sigma-Aldrich Corporation, Beijing local agent, China), cholesterol (Sigma-Aldrich Corporation, Beijing local agent, China), polyethylene glycol-distearoylphosphatidylethanolamine (PEG_{2000}-DSPE, Avanti Polar Lipids, Alabaster, AL, USA) and octadecylamine (Johnson Matthey Company, London, UK) (54/23/4/2.5, μmol ratio) were dissolved in chloroform in a pear-shaped flask. The chloroform was evaporated to dryness under vacuum with a rotary evaporator, and then the formed lipid film was hydrated with 250 mM ammonium sulfate by sonication in the water bath for 5 min, followed by sonication using a probe-type sonicator for 10 min. The suspensions after hydration were successively extruded through polycarbonate membranes (Millipore, Bedford, MA, USA) with the pore size of 400 nm, and 200 nm for 3 times, respectively. Afterwards, the blank PEG-coated cationic liposomes were obtained[19].

To prepare PEG-coated irinotecan cationic liposomes, the blank PEG-coated cationic liposomes were then dialyzed (8,000-15,000 molecular mass cutoff) (Beijing Dingguo Biotechnology Limited Company, China) in the 500 ml physiological saline (0.9%NaCl) for two times. Irinotecan was loaded using an ammonium sulfate gradient loading method, as reported previously. Appropriate amounts of irinotecan hydrochloride (Jiangsu Henrui Medicine Corporation, China) were added to the blank PEG-coated cationic liposomes. After mixing, the suspensions were incubated at 50 ºC in water bath, and intermittently shaken for 30 min to produce the PEG-coated irinotecan cationic liposomes.

To prepare PEG-coated irinotecan liposomes, the blank PEG-coated liposomes were made with the same procedures of blank PEG-coated cationic liposomes, excluding the addition of octadecylamine during film forming. Then irinotecan was loaded into the blank PEG-coated liposomes using an ammonium sulfate gradient loading method as above.

Characterization of PEG-coated Irinotecan Cationic Liposomes

PEG-coated irinotecan cationic liposomes and PEG-coated irinotecan liposomes were passed over a Sephadex G-50 column (Sigma-Aldrich Corporation, Beijing local agent, China) to remove the free irinotecan. The encapsulation efficiency of irinotecan was calculated with the formula: $EE = (W_{encap}/W_{total}) \times 100\%$, where EE is the encapsulation efficiency of irinotecan, and W_{encap} is the measured amount irinotecan in the liposomal suspensions after passing over the column. The irinotecan concentration was determined by using fluorospectrophotometer.

In vitro release of irinotecan in the liposomes was performed by the dialysis against the release medium containing serum protein (phosphate buffered saline containing 10% fetal calf serum). A volume of 2.5 ml liposomes plus 2.5 ml of release medium in dialysis tubing was immersed in 30.0 ml of the release medium, and oscillated with a shaker at a rate of 150 times per minute at 37 ºC. A volume of 0.5 ml release medium was taken at 0, 0.25, 0.5, 1, 2, 4, 6, 8, 12, 24 and 48 h, respectively, and immediately replaced with the same volume of fresh release medium after each sampling. The irinotecan content in the release medium was determined by fluorospectrophotometer as above. The release rate was calculated with the formula: $RR = (W_i/W_{total}) \times 100\%$, where RR is the drug release rate (%), W_i is the measured amount of irinotecan at the time-point of ith h in release medium, and W_{total} is the total amount of irinotecan in the equal volume of liposome suspensions prior to dialysis[20].

The particle sizes and zeta potential values of all nanovesicles were measured with Zetasizer 3000HSA (Malvern Instruments Ltd., Malvern, Worcestershire, UK).

Culture of MDA-MB231 Cells

The culture medium was prepared with DMEM (Sigma-Aldrich Corporation, Beijing local agent, China) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich Corporation, Beijing local agent, China), 100 units/ml penicillin (Sigma-Aldrich Corporation, Beijing local agent, China), and 100 units/ml streptomycin (Sigma-Aldrich Corporation, Beijing local agent, China). The cells were cultured in the incubator at 37°C and in the presence of 5% CO_2.

Cytotoxicity to MDA-MB231 Cells

MDA-MB231 cells were seeded into 96-well culture plates at a density of 9.0×10^3 cells per well

and grown in culture medium in the incubator at 37°C and in the presence of 5% CO_2 for 24 h. Free irinotecan (0-5 μM), PEG-coated irinotecan liposomes (0-5 μM) and PEG-coated irinotecan cationic liposomes (0-5 μM) were added into 96-well culture plates, respectively. The survival rate was measured at 48 h by the sulforhodamine B (SRB) staining assay, and the absorbance was read on a microplate reader at 540 nm. The survival percentages were calculated using the following formula: Survival % = (A_{540nm} for the treated cells/$A_{540\ nm}$ for the control cells) × 100%, where $A_{540\ nm}$ is the absorbance value. Each assay was repeated in triplicate. Finally, the dose-effect curves were plotted.

Intracellular Uptake of PEG-coated Irinotecan Liposomes

Intracellular uptake of PEG-coated irinotecan liposomes was determined by flow cytometry assay. MDA-MB231 cells were seeded onto six-well plates at 3.0×10^5 per well and cultured for 24 h at 37°C and in the presence of 5% CO_2, followed by adding PEG-coated irinotecan liposomes (2.5 μM) and PEG-coated irinotecan cationic liposomes (2.5 μM). Phosphate buffered saline (PBS) (pH 7.4) was added as a blank control. The cells were further incubated for 5 h and then enzymatically dissociated by adding trypsin (0.25%, g/100 ml). Cells were resuspended in PBS (pH 7.4). The samples were determined by flow cytometry with a FACScan (Becton Dickinson, San Jose, CA, USA). Data were collected of 10,000 gated events and analyzed with the CELL Quest software program.

Morphology of Nuclei Induced by PEG-coated Irinotecan Cationic Liposomes

Hoechst staining of nuclei was performed to observe morphological changes of nuclei in MDA-MB231 cells. Briefly, MDA-MB231 cells were seeded at 3.8×10^4 cells per well in 24-well plates and cultured in the incubator at 37°C and in the presence of 5% CO_2 for 24 h, then treated with free irinotecan (2.5 μM), PEG-coated irinotecan liposomes (2.5 μM) and PEG-coated irinotecan cationic liposomes (2.5 μM), and further incubated for 6 h. Afterwards, cells were fixed with 4% paraformaldehyde for 0.5 h and stained with Hoechst 33342 (120 mg/ml) for 25 min. Cells were examined with a fluorescence microscope (Leica, Heidelberg, Germany) for observing the fragmentation of nuclei. Cells were treated with PBS (pH 7.4) as control.

Animals

For each optimized PEG-coated irinotecan cationic liposomes studied, female BALB/c nude mice and Sprague-Dawley (SD) rats were obtained from Vital Laboratory Animal Center of Hebei Medical University. All of the animal experiments adhered to the principles for care and use of laboratory animals and were approved by the institutional Animal Care and Use Committee of Hebei Medical University.

In vivo Inhibition of the Tumor Growth and Effects on the Indicators of Bone Marrow

Female BALB/c nude mice, initially weighing 18-20 g, were used for investigating the antitumor efficacy *in vivo*. Briefly, approximately 2.0×10^7 MDA-MB231 cells were resuspended in 100 μl of serum-free medium, and injected subcutaneously into the right flanks of the nude mice[21]. When tumors reached 100 to 150 mm^3 in volume, mice were randomly divided into four treatment groups (6 for each group). At day 19, 22, 25, and 28 post inoculation, physiological saline (blank control), free irinotecan (20.00 mg/kg), PEG-coated irinotecan liposomes (20.00 mg/kg), and PEG-coated irinotecan cationic liposomes (20.00 mg/kg) were given intravenously to mice which had been randomly divided into four treatment groups (6 for each) via tail vein, respectively. The presence of each tumor mass was confirmed by necropsy at day 31 since the inoculation. Mice were weighed and tumors were measured with a caliper every one or every two days. Tumor volumes were calculated with the formula (length × $width^2$/2).

At day 31, the mice were sacrificed. Blood specimens of the mice were collected immediately. The blood specimens were used for measuring the indicators of bone marrow in peripheral blood (white blood cells, WBC; hemoglobin, Hb; platelet, PLT).

Pharmacokinetics and Tissue Distribution

Female Sprague-Dawley (SD) rats were used for investigating the pharmacokinetics and randomly divided into three groups (6 for each group). Free irinotecan (20.00 mg/kg), PEG-coated irinotecan liposomes (20.00 mg/kg) and PEG-coated irinotecan cationic liposomes (20.00 mg/kg) were given intravenously to rats via tail vein, respectively. After administrations, a 1ml volume of plasma was collected in a heparinized microcentrifuge tube from retro orbital sinus at the

following time points: 15 min, 30 min, 1h, 2h, 4h, 8h, 12h, 24h and 48h. The plasma was separated by centrifugation at 12,000 rpm for 10 min and stored at –20°C [2]. A 3.0-ml volume of methanol was added to the 100 µl plasma and mixed for 3 min with a vortex, followed by centrifugation at 10,000 rpm for 5 min. The liquid phase was transferred to a clean tube and dried under a gentle N_2 gas stream to obtain residues containing the irinotecan. The irinotecan residue was reconstituted with 100 µl mobile phase and centrifuged at 10,000 rpm for 5 min. Finally, a 20 µl volume of supernatant was injected into the HPLC system.

Female BALB/c nude mice were used for investigating the tissue distribution after xenografting MDA-MB231 breast cancer cells and randomly divided into three groups (6 for each group). Free irinotecan (20.00 mg/kg), PEG-coated irinotecan liposomes (20.00 mg/kg) and PEG-coated irinotecan cationic liposomes (20.00 mg/kg) were given intravenously to mice via tail vein, respectively. After administration, heart, liver, spleen, lung, kidney and tumor were also collected at 1h, 4h, 8h, 24h and 48h after sacrifice by cervical dislocation. The tissues were washed with physiological saline, dried with filter paper, weighted accurately and stored at –20°C [23]. The tissue samples were homogenized with a homogenizer in physiological saline. A 3.0-ml volume of methanol was added to 100 µl tissue homogenate (0.15 g of tissue) and mixed for 3 min with a vortex, followed by centrifugation at 10,000 rpm for 5 min. The liquid phase was transferred to a clean tube and dried under a gentle N_2 gas stream to obtain residues containing the irinotecan. The irinotecan residue was reconstituted with 100 µl mobile phase and centrifuged at 10,000 rpm for 5 min. Finally, a 20 µl volume of supernatant was injected into the HPLC system.

The samples were analyzed by HPLC with fluorescence detection (Waters Technologies Inc., Cotati, CA, USA). Irinotecan was separated by a Diamonsil C_{18} column (200×4.6 mm, 5 µm). The mobile phase consisted of acetonitrile and water containing 2.1% citric acid and 0.2% triethylamine (40/60, v/v) with a flow rate of 1.0 ml/min under isocratic conditions. pH was 3.8 settled by 30% natrium hydroxydatum. The fluorescence detection was set at λ_{ex} 370 nm and λ_{em} 530 nm.

Statistical Analysis

Data are presented as the mean ± standard deviation. One-way analysis of variance was used to determine significance among groups, after which post hoc tests with the Bonferroni correction were used for multiple comparisons between individual groups. A value of $p < 0.05$ was considered to be significant.

Results

Characterization of PEG-coated Irinotecan Cationic Liposomes

Table I represents the characterization results of two liposomes. In two kinds of liposomes prepared, the encapsulation efficiency of irinotecan was ≥ 90%. The mean particle sizes of PEG-coated irinotecan liposomes and PEG-coated irinotecan cationic liposomes were 68.65 ± 5.19 and 66.32 ± 3.06 nm (Figure 1B), respectively. Their corresponding potential val-

Table I. Characterizations of two irinotecan liposomes.

1. Encapsulation efficiency of irinotecan	Results (%)
PEG-coated irinotecan liposomes	93.66 ± 3.12%
PEG-coated irinotecan cationic liposomes	91.39 ± 2.58%
2. Size of irinotecan liposomes	**Results**
	Mean size (nm)
PEG-coated irinotecan liposomes	68.65 ± 5.19
PEG-coated irinotecan cationic liposomes	66.32 ± 3.06
3. Zeta potential of irinotecan liposomes	**Results**
PEG-coated irinotecan liposomes	–15.19 ± 0.68 mv
PEG-coated irinotecan cationic liposomes	23.13 ± 0.59 mv

Each point represents means ± SD (n=3).

ues were -15.19±0.68 mV and 23.13±0.59 mV, respectively.

Figure 1C shows the release rates of irinotecan from two different liposomes in PBS solution containing 10% serum protein oscillated at a rate of 150 times per minute at 37ºC. Release rate of irinotecan from the two kinds of liposomes was negligible under the same release condition. The release rates at 48 h of irinotecan from PEG-coated irinotecan liposomes and PEG-coated irinotecan cationic liposomes were 29.92 ± 0.81% and 28.20 ± 1.36%, respectively.

Cytotoxicity to MDA-MB231 Cells

Figure 2A shows the inhibitory effects to MDA-MB231 cells after applying various formulations. As compared to free irinotecan and PEG-coated irinotecan liposomes, PEG-coated irinotecan cationic liposomes showed the strongest inhibitory effects at various dose levels. For example, the rank of survival rates after applying 5 μM irinotecan were PEG-coated irinotecan liposomes (23.77 ± 1.43%) > free irinotecan (5.57 ± 1.14%) > PEG-coated irinotecan cationic liposomes (3.48 ± 1.01%).

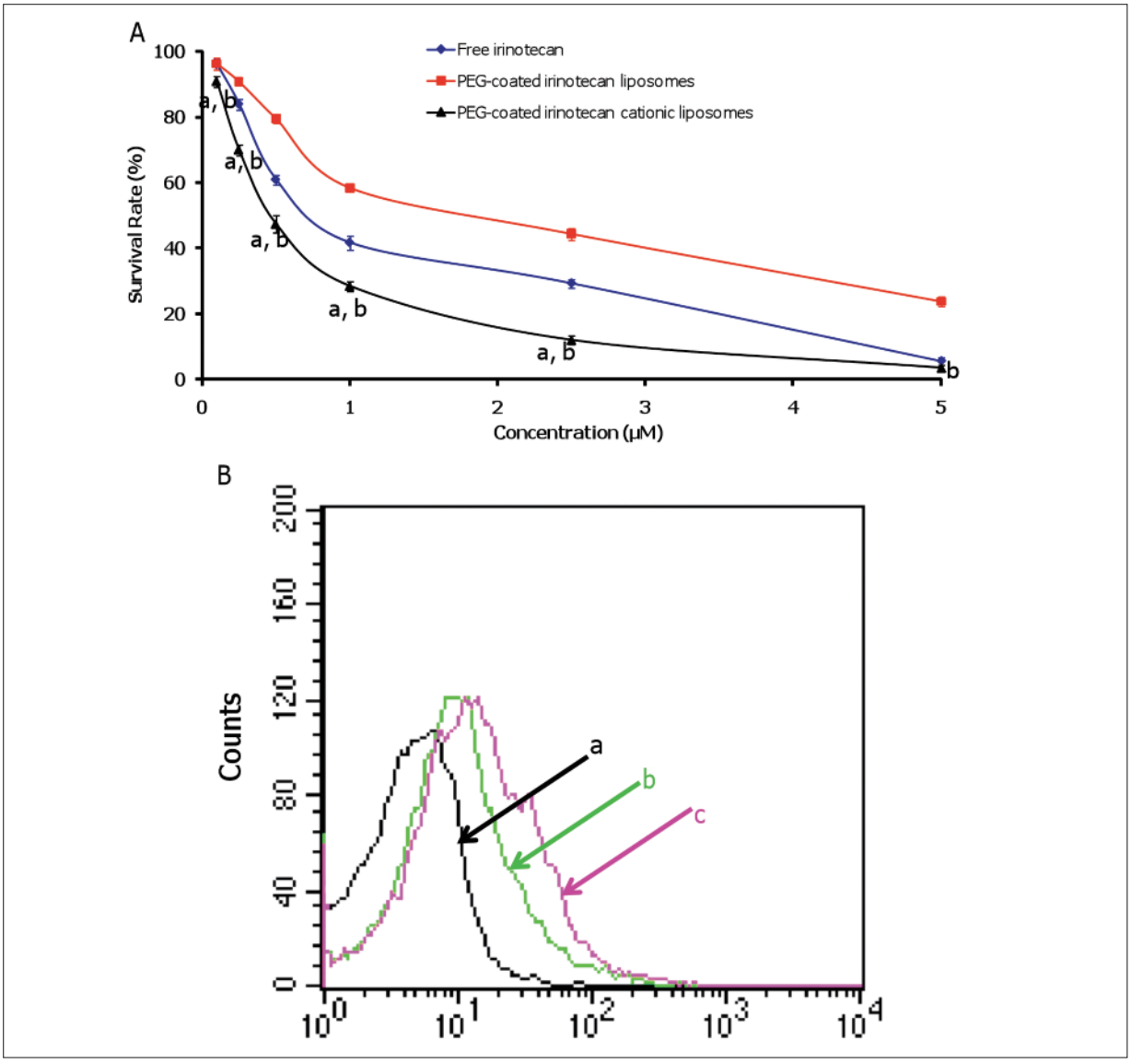


Figure 2. A, Survival rates of MDA-MB-231 cells after applying various irinotecan formulations measured by SRB staining assay. Data are presented as the mean ± SD (n=3). a, $p < 0.05$, versus free irinotecan; b, $p < 0.05$, versus PEG-coated irinotecan liposomes. ***B,*** MDA-MB-231 cellular uptakes after applying various irinotecan formulations measured by FAScan flow cytometry. Notes: a, PBS; b, PEG-coated irinotecan liposomes; c, PEG-coated irinotecan cationic liposomes.

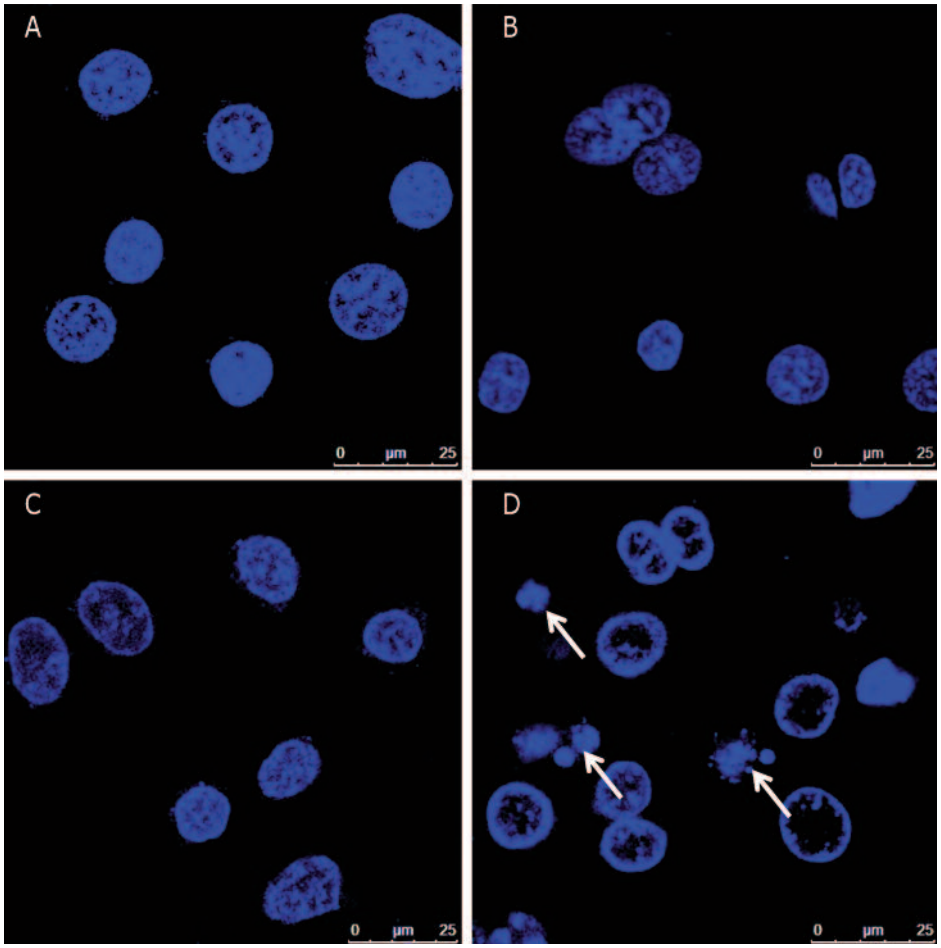


Figure 3. Image of the nuclear morphological changes of MDA-MB-231 induced by applying various formulations, including PBS as blank control ***(A)***, Free irinotecan ***(B)***, PEG-coated irinotecan liposomes ***(C)*** and PEG-coated irinotecan cationic liposomes ***(D)***, respectively. Arrows: fragmented nuclei.

Intracellular Uptake of PEG-coated Irinotecan Liposomes

Figure 2B shows semi-quantitative drug content observed by flow cytometry in the MDA-MB231 cells after applying PEG-coated irinotecan liposomes and PEG-coated irinotecan cationic liposomes at 5 h.

In quantitative evaluation for ten thousands of events, the geometric mean intensity value was 8.62 for PBS (a) < 15.36 for PEG-coated irinotecan liposomes (b) <22.19 for PEG-coated irinotecan cationic liposomes (c).

Morphology of Nuclei Induced by PEG-Coated irinotecan Cationic Liposomes

Figure 3 shows images for nuclei of MDA-MB231 cells after applying free irinotecan, PEG-coated irinotecan liposomes and PEG-coated irinotecan cationic liposomes.

After applying PEG-coated irinotecan cationic liposomes, nuclei of MDA-MB231 cells became broken and evident fragment occurred as early as 6 h, showing characteristics of nuclei as MDA-MB231 breast cancer cells death. As compared to free irinotecan and PEG-coated irinotecan liposomes, PEG-coated irinotecan cationic liposomes showed the strongest inhibition effect of MDA-MB231 cancer cells.

In vivo Inhibition of the Tumor Growth and Effects on the Indicators of Bone Marrow

Figure 4A shows the efficacy of PEG-coated irinotecan cationic liposomes in treating the MDA-MB231 cells xenografts tumor model. After inoculation of MDA-MB231 cells in nude mice, the tumors reached suitable masses for treatment at day 19. As compared to blank control group, the inhibitory effects of tumor growth were obviously observed in all treatment groups. The rank of inhibitory effects was PEG-coated irinotecan cationic liposomes > PEG-coated irinotecan liposomes > free irinotecan > the blank control.

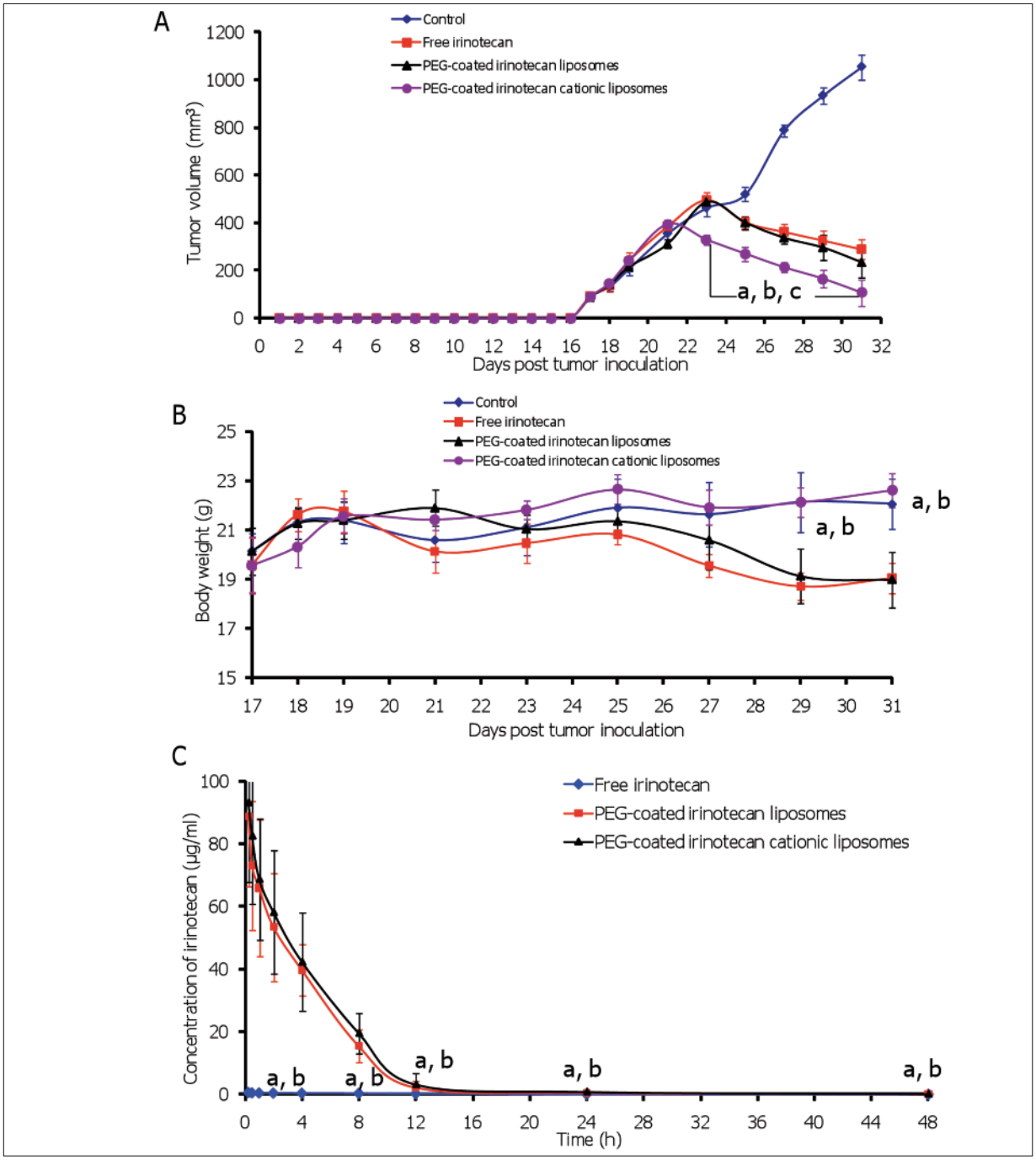


Figure 4. A, Effect of PEG-coated irinotecan cationic liposomes on the MDA-MB-231 cells xenografts in female nude mice. At day 19, 22, 25 and 28 after inoculation, physiological saline as control group, free irinotecan (20.00 mg/kg), PEG-coated irinotecan liposomes (20.00 mg/kg), and PEG-coated irinotecan cationic liposomes (20.00 mg/kg) were given intravenously to mice via tail vein, respectively. Data are presented as the mean ± SD (n=6). a, $p < 0.05$, versus physiological saline; b, $p < 0.05$, versus free irinotecan; c, $p < 0.05$, versus PEG-coated irinotecan liposomes. ***B,*** Body weight changes for the tumor-bearing mice after intravenous administration of physiological saline, free irinotecan, PEG-coated irinotecan liposomes and PEG-coated irinotecan cationic liposomes to mice. Data are presented as the mean ± standard deviation (n=6). a, $p < 0.05$, versus free irinotecan; b, $p < 0.05$, versus PEG-coated irinotecan liposomes. ***C,*** Time course of irinotecan levels in plasma after intravenous administration of free irinotecan (20.00 mg/kg), PEG-coated irinotecan liposomes (20.00 mg/kg) and PEG-coated irinotecan cationic liposomes (20.00 mg/kg). Each point represents means ± SD (n=6). a, $p < 0.05$, versus PEG-coated irinotecan liposomes; b, $p < 0.05$, versus PEG-coated irinotecan cationic liposomes.

Table II. Effect on the hematological indicators of bone marrow after intravenous administration of physiological saline (blank control), free irinotecan, PEG-coated irinotecan liposomes and PEG-coated irinotecan cationic liposomes in female BALB/c nude mice.

Indicator	Blank control	Free irinotecan	PEG-coated irinotecan liposomes	PEG-coated irinotecan cationic liposomes
WBC (10^9/L)	9.63 ± 0.53	6.01 ± 0.56[a]	8.59 ± 0.72[a]	9.43 ± 0.81
Hb (g/L)	106.33 ± 9.12	75.26 ± 11.13[a]	91.55 ± 10.02[a]	101.29 ± 11.23
PLT (10^9/L)	988.65 ± 35.69	633.61 ± 48.12[a]	901.31 ± 57.42[a]	971.32 ± 39.61

Data are presented as means ± SD (n = 6). WBC: white blood cell; Hb: hemoglobin; PLT: platelets. [a]$p < 0.05$, versus blank control.

Figure 4B shows the body weight changes of the tumor-bearing mice during the study of anti-tumor efficacy. In view of results observed in all treatment groups, body weight loss was not observed significantly in the mice after giving PEG-coated irinotecan cationic liposomes but found in the mice after giving PEG-coated irinotecan liposomes and free irinotecan.

Table II shows the effects of various formulations on the hematological indicators of bone marrow. After administration of PEG-coated irinotecan cationic liposomes, the levels of WBC, Hb, and PLT in blood specimens were slightly decreased, but without significant difference as compared to those in control group. After administration of PEG-coated irinotecan liposomes, the levels of WBC, Hb, and PLT in blood specimens were decreased. However, the levels of WBC, Hb, and PLT were severely lowered after administration of free irinotecan.

Pharmacokinetics of Irinotecan in Plasma

After administration of free irinotecan, the plasma irinotecan level dropped rapidly. While after administration of PEG-coated irinotecan liposomes and PEG-coated irinotecan cationic liposomes, the plasma irinotecan concentration decreased relatively slowly in the initial phase and remained at higher concentration levels in the terminal phase (Figure 4C), resulting in longer blood exposure.

The pharmacokinetic parameters were calculated according to a two-compartment model (Table III).The order of AUC in plasma was: PEG-coated irinotecan cationic liposomes (312.93±62.49 $\mu g \cdot h \cdot ml^{-1}$) ≥ PEG-coated irinotecan liposomes (299.79± 58.91 $\mu g \cdot h \cdot ml^{-1}$) > free irinotecan (8.97 ± 2.73 $\mu g \cdot h \cdot ml^{-1}$). With respect to the elimination half-life in the terminal phase, the rank order of $t_{1/2}$ β values in plasma was: PEG-coated irinotecan cationic liposomes (21.71 ± 5.41h^{-1}) ≥ PEG-coated irinotecan liposomes (19.66 ± 6.35h^{-1}) > free irinotecan (13.29 ± 6.94h-1). The $t_{1/2}$ α value of PEG-coated irinotecan cationic liposomes was longer than that of free irinotecan but similar to that of PEG-coated irinotecan liposomes. The C_{max} and MRT value of free irinotecan were significantly decreased relative to PEG-coated irinotecan liposomes and PEG-coated irinotecan cationic li-

Table III. Pharmacokinetics of irinotecan after intravenous administration of free irinotecan, PEG-coated irinotecan liposomes and PEG-coated irinotecan cationic liposomes in female rats at a dose of 20.00 mg/kg.

Pharmacokinetic parameters	Free irinotecan	PEG-coated irinotecan liposomes	PEG-coated irinotecan cationic liposomes
$t_{1/2}$ α (h)	1.65 ± 0.82	1.93 ± 0.22	1.88 ± 0.39
$t_{1/2}$ β (h)	13.29 ± 6.94	19.66 ± 6.35	21.71 ± 5.41
C_{max} (μg/ml)	0.91 ± 0.33	89.52 ± 18.33	93.26 ± 20.03
CL (ml/h/kg)	1.85 ± 0.29	0.05 ± 0.01	0.04 ± 0.01
$MRT_{0\text{-}48h}$ (h)	9.85 ± 3.51	14.22 ± 0.68	15.86 ± 1.12
$AUC_{0\text{-}48h}$ (μg·h/ml)	8.97 ± 2.73	299.79 ± 58.91	312.93 ± 62.49

Data are presented as means ± SD (n = 6). $t_{1/2}$α (h), distribution half life; $t_{1/2}$ β (h), elimination half life; C_{max}, peak concentration; CL (ml/h/kg), total body clearance; $MRT_{0\text{-}48h}$, mean residence time; $AUC_{0\text{-}48h}$ (μg/ml·h), area under the plasma concentration-time curve.

posomes. But the CL value of free irinotecan was higher than those of PEG-coated irinotecan liposomes and PEG-coated irinotecan cationic liposomes.

Tissue Distribution of Irinotecan

Figure 5 and Table IV present the irinotecan levels in heart, liver, spleen, lung, kidney and tumor after intravenous administration of free irinotecan, PEG-coated irinotecan liposomes and PEG-coated irinotecan cationic liposomes at 1.0 h, 4.0 h, 8.0 h, 24 h and 48 h.

In heart, lung and kidney tissues, the irinotecan levels were approximately similar treated with free irinotecan, PEG-coated irinotecan liposomes and PEG-coated irinotecan cationic liposomes. But in liver and spleen tissue, the irinotecan level was lower in animals treated with PEG-

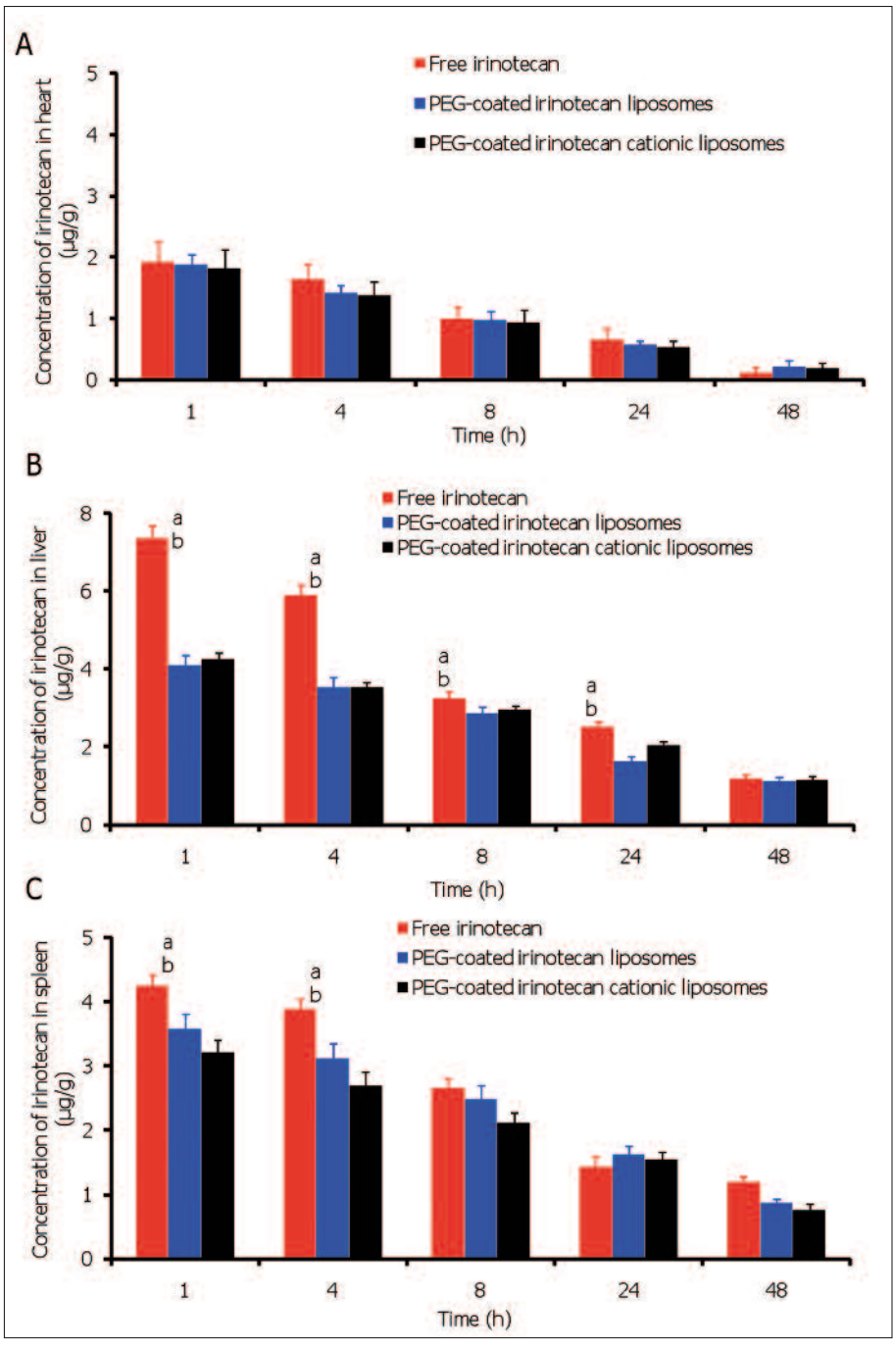
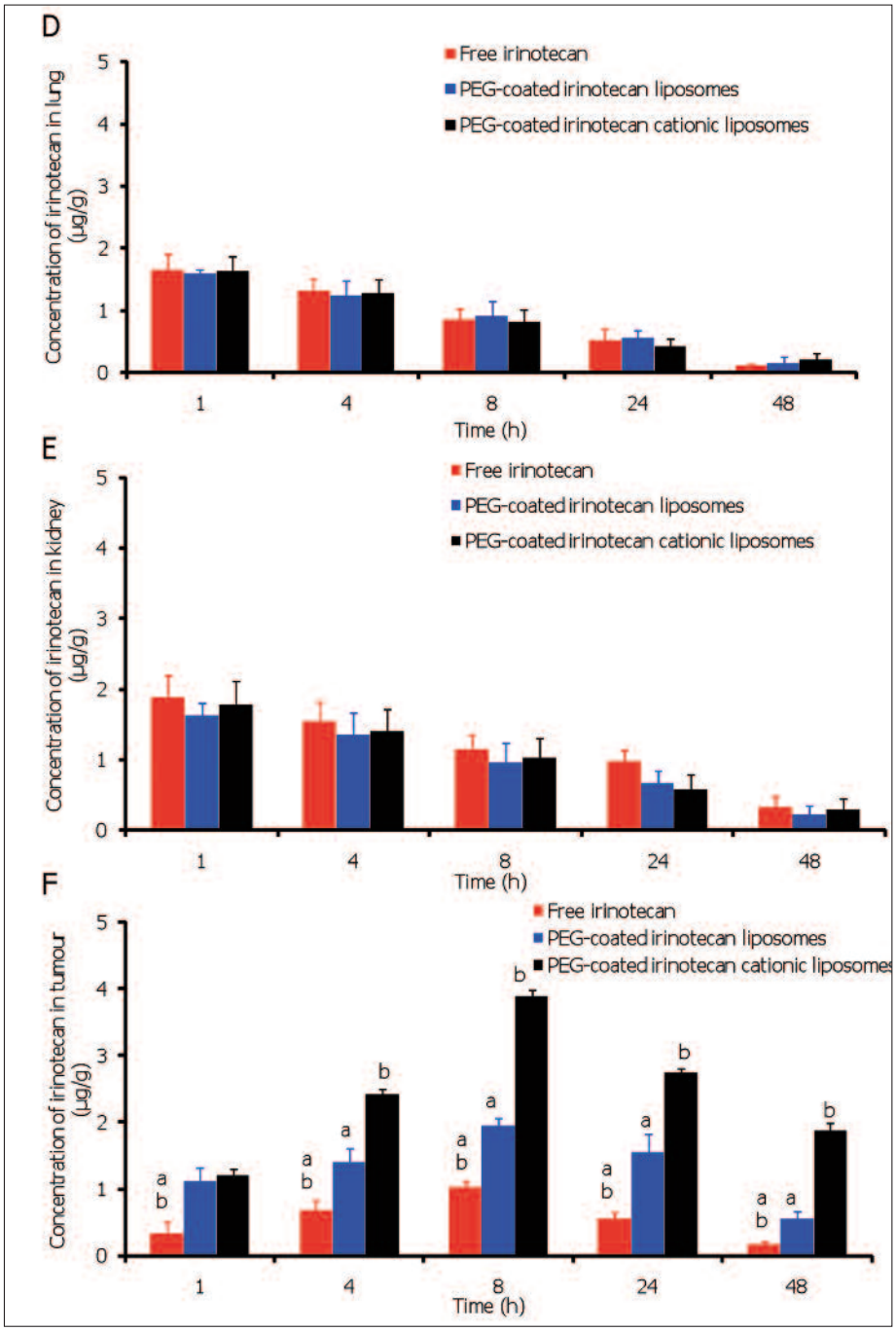


Figure 5. Irinotecan level in heart ***(A)***, liver ***(B)***, spleen ***(C)***, lung ***(D)***, kidney ***(E)*** and tumor ***(F)*** after intravenous injection of free irinotecan, PEG-coated irinotecan liposomes and PEG-coated irinotecan cationic liposomes in female BALB/c nude mice. Each point represents means ± SD (n = 6). a, $p < 0.05$, versus PEG-coated irinotecan cationic liposomes; b, $p < 0.05$, versus PEG-coated irinotecan liposomes.

Continues in the next page

Figure 5. *Continued.*



coated irinotecan cationic liposomes and PEG-coated irinotecan liposomes than in those treated with free irinotecan. The rank order of AUC values in liver tissue was: free irinotecan (81.56±3.73 ng·h/g) > PEG-coated irinotecan cationic liposomes (55.21±2.26 ng·h/g) ≥ PEG-coated irinotecan liposomes (49.91±3.51 ng·h/g). The rank order of AUC values in spleen tissue was: free irinotecan (51.82±3.04 ng·h/g) > PEG-coated irinotecan liposomes (45.56±3.89 ng·h/g) ≥ PEG-coated irinotecan cationic liposomes (39.72±3.14 ng·h/g).

In tumor masses (Figure 5F), the irinotecan level was significantly higher in the mice treated with PEG-coated irinotecan cationic liposomes than in those treated with free irinotecan and PEG-coated irinotecan liposomes at the 4, 8, 24 and 48h time points. The rank order of AUC_{0-48h} values in tumor was: PEG-coated irinotecan cationic liposomes (59.24±1.87 ng·h/g) > PEG-

Table IV. AUC0–48h (μg·h/g) after intravenous administration of free irinotecan, PEG-coated irinotecan liposomes and PEG-coated irinotecan cationic liposomes in female BALB/c nude mice at a dose of 20.00 mg/kg (n = 6).

Tissue	Free irinotecan	PEG-coated irinotecan liposomes	PEG-coated irinotecan cationic iposomes
Heart	18.71 ± 4.18	17.63 ± 2.34	16.78 ± 3.39
Liver	81.56 ± 3.73[ab]	49.91 ± 3.51	55.21 ± 2.26
Spleen	51.82 ± 3.04[ab]	45.56 ± 3.89	39.72 ± 3.14
Lung	15.46 ± 3.25	15.80 ± 3.33	14.88 ± 3.41
Kidney	22.72 ± 4.35	16.69 ± 4.64	18.19 ± 3.30
Tumor	12.49 ± 2.08[ab]	30.20 ± 4.17[a]	59.24 ± 1.87[b]

Data are presented as means±SD. [a]$p < 0.05$, versus PEG-coated irinotecan liposomes; [b]$p < 0.05$, versus PEG-coated irinotecan cationic liposomes.

coated irinotecan liposomes (30.20±4.17 ng·h/g) > free irinotecan (12.49±2.08 ng·h/g).

Discussion

Nowadays, research into the rational delivery and targeting of pharmaceutical agents is at the forefront of projects in cancer treatment. These treatments based nanoparticles can improve the specificity and help treatment cancer that cannot be treated with traditional ways, provide more effective and more convenient routes of administration, lower therapeutic toxicity, extend the product life cycle, and ultimately reduce health-care costs[24,25]. In the past few years, a number of nanoparticle-based therapeutic agents have been developed for the treatment of cancer. As a complicated therapeutic delivery system, liposome is one of the most successful nanoparticles in the market approved by FDA for the chemotherapeutic drug loading and delivery[26,27].

In this study, a kind of PEG-coated irinotecan cationic liposomes was developed for treating breast cancer. Polyethylene glycol (PEG_{2000}-DSPE) has been used widely in nanoparticle formulations as a strategy to inhibit opsonization by plasma proteins and to prolong nanoparticle plasma circulation time. The specified uptake of PEG-coated irinotecan cationic liposomes probably relies on electrostatic interaction with negatively charged target structures on MDA-MB231 breast cancer cells membrane[28]. Among the cancer cells associated target structures, there are negatively charged cell surface molecules such as glycoproteins, anionic phospholipids and proteoglycans. These are potential binding sites for cationic liposomes[29,30]. The selectivity of PEG-coated irinotecan cationic liposomes offers the possibility of targeted delivery of irinotecan or other therapeutic agents to tumor cells.

Results demonstrate that PEG-coated irinotecan cationic liposomes have the following physicochemical features: high encapsulation efficiency (Table I), well-distributed particle size (Figure 1B), and delayed drug release (Figure 1C). A lower drug release will be beneficial for preventing the rapid leakage during the process of delivery and blood/lymphatic circulation of PEG-coated irinotecan cationic liposomes, thereby possibly increasing the accumulation of the irinotecan into the tumor masses.

Cytotoxicity results demonstrate that the PEG-coated irinotecan cationic liposomes exhibit strong inhibitory effect to MDA-MB231 cancer cells (Figure 2A). The enhanced inhibitory effects of the PEG-coated irinotecan cationic liposomes are explained by MDA-MB231 cancer cells uptake study in which the PEG-coated irinotecan cationic liposomes are the most strongly endocytosed by MDA-MB231 cancer cells. In a semi-quantification way, flow cytometric measurement gives the relative drug content in the cancer cells. Results show that the PEG-coated irinotecan cationic liposomes evidently increase the irinotecan content in the MDA-MB231 breast cancer cells (Figure 2B).

The morphological studies demonstrate the existence of the nuclei fragment induced by PEG-coated irinotecan cationic liposomes. In view of the fluorescent microscopic images stained by chromatin dye Hoechst 33342 (Figure 3), the irinotecan binds to the topoisomerase I-DNA complex, thereby preventing re-ligation of DNA breaks resulting in the formation of irreversible double-strand breaks and existence of the nuclei fragment after applying PEG-coated irinotecan cationic liposomes, showing the change of nuclei as MDA-MB231 cancer cells death[31].

The inhibitory effects on tumor volume in the MDA-MB231 breast cancer cells xenografted female nude mice demonstrate that, by intravenous injection administration, the PEG-coated irinotecan cationic liposomes exhibit significantly higher antitumor activity as compared to PEG-coated irinotecan liposomes and free irinotecan (Figure 4A). As compared to free irinotecan and PEG-coated irinotecan liposomes, PEG-coated irinotecan cationic liposomes do not cause an appreciable reduction in body weight (Figure 4B).

After administering PEG-coated irinotecan cationic liposomes to the MDA-MB231 breast cancer cells xenografted female nude mice, the bone marrow indicators (WBC, Hb, and PLT) are mildly decreased, suggesting that PEG-coated irinotecan cationic liposomes may have no significant influences on bone marrow (Table II). At least, the potential myelosuppression effect may be no more than those after administering free irinotecan and PEG-coated irinotecan liposomes. Gastrointestinal toxicity (diarrhea) is frequent observed after administration of free irinotecan. It was controlled with the administration of PEG-coated irinotecan cationic liposomes and PEG-coated irinotecan liposomes.

When comparing the concentration-time profile of free irinotecan with that of PEG-coated irinotecan liposomes or PEG-coated irinotecan cationic liposomes, irinotecan blood exposure was clearly extended by the pegylated liposomes, showing a long-circulatory effect[32,33]. Pegylation with PEG_{2000}-DSPE prevents adsorption of opsonin proteins onto the surface of irinotecan liposomes, thereby prolonging the circulation time of the liposomes by avoiding rapid uptake by the reticuloendothelial system (Figure 4C).

The liver and spleen are major sites of drug metabolism and are rich in reticuloendothelial cells[34,35]. No significant difference in irinotecan levels was detected between animals administered PEG-coated irinotecan cationic liposomes and those given PEG-coated irinotecan liposomes. But after administration of PEG-coated irinotecan cationic liposomes or PEG-coated irinotecan liposomes, the irinotecan level in liver and spleen tissue was lower than free irinotecan after administration. This may be explained by the pegylated liposomes escaping rapid uptake by the reticuloendothelial system in the liver and spleen (Figure 5B and C).

Administering PEG-coated irinotecan cationic liposomes resulted in higher drug concentrations in the tumor masses than the other formulations. The higher level of irinotecan in the tumor could be attributed to specificity bind of PEG-coated irinotecan cationic liposomes by electrostatic interaction (Figure 5F). Furthermore, PEG-coated irinotecan cationic liposomes demonstrate a robust anticancer activity against the tumor xenografted with MDA-MB231 breast cancer cells.

Conclusions

In the present work, the PEG-coated irinotecan cationic liposomes were successfully developed with high entrapped efficiency, perfect size distribution, and slow releasing. The efficacies are confirmed *in vitro* and in the breast tumor by xenografting MDA-MB231 breast cancer cells into female nude mice. Mechanism studies demonstrate that PEG-coated irinotecan cationic liposomes improve the circulation time of irinotecan in the blood by escaping rapid uptake by the reticuloendothelial system and specificity bind into tumor masses by electrostatic interaction with negatively charged target structures on breast cancer cells membrane, resulting in enhancing the concentration of irinotecan in tumor area and exhibiting strong inhibitory effect to breast tumor volume. The present study provides a new strategy for treatment of the breast cancer.

Statement of Interest

This study was funded by the Colleges and Universities in Hebei Province Science and Technology Research Project (No. ZD200907).

Conflict of Interest

The Authors declare that there are no conflicts of interest.

References

1) Burns MB, Lackey L, Carpenter MA, Rathore A, Land AM, Leonard B, Refsland EW, Kotandeniya D, Tretyakova N, Nikas JB, Yee D, Temiz NA, Donohue DE, McDougle RM, Brown WL, Law EK, Harris RS. APOBEC3B is an enzymatic source of mutation in breast cancer. APOBEC3B is an enzymatic source of mutation in breast cancer. Nature 2013; 494: 366-370.

2) Montagner M, Enzo E, Forcato M, Zanconato F, Parenti A, Rampazzo E, Basso G, Leo G, Rosato A, Bicciato S, Cordenonsi M, Piccolo S. SHARP1 suppresses breast cancer metastasis by promoting degradation of hypoxia-inducible factors. Nature 2012; 487: 380-384.

3) Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011; 61:69-90.

4) Rexer BN, Arteaga CL. Optimal targeting of HER2-PI3K signaling in breast cancer: mechanistic insights and clinical implications. Cancer Res 2013; 73: 3817-3820.

5) Heinzel A, Müller D, Langen KJ, Blaum M, Verburg FA, Mottaghy FM, Galldiks N. The use of O-(2-18F-fluoroethyl)-L-tyrosine PET for treatment management of bevacizumab and irinotecan in patients with recurrent high-grade glioma: a cost-effectiveness analysis. J Nucl Med 2013; 54: 1217-1222.

6) Takano M, Goto T, Hirata J, Furuya K, Horie K, Takahashi M, Yokota H, Kino N, Kudoh K, Kikuchi Y. UGT1A1 genotype-specific phase I and pharmacokinetic study for combination chemotherapy with irinotecan and cisplatin: a Saitama Tumor Board study. Eur J Gynaecol Oncol 2013; 34: 120-123.

7) Raciborska A, Bilska K, Drabko K, Chaber R, Pogorzala M, Wyrobek E, Polczy ska K, Rogowska E, Rodriguez-Galindo C, Wozniak W. Vincristine, irinotecan, and temozolomide in patients with relapsed and refractory Ewing sarcoma. Pediatr Blood Cancer 2013; 60: 1621-1625.

8) Jones RP, Sutton P, Greensmith RM, Santoyo-Castelazo A, Carr DF, Jenkins R, Rowe C, Hamlett J, Park BK, Terlizzo M, O'Grady E, Ghaneh P, Fenwick SW, Malik HZ, Poston GJ, Kitteringham NR. Hepatic activation of irinotecan predicts tumour response in patients with colorectal liver metastases treated with DEBIRI: exploratory findings from a phase II study. Cancer Chemother Pharmacol 2013; 72: 359-368.

9) Tsubamoto H, Kawaguchi R, Ito K, Shiozaki T, Takeuchi S, Itani Y, Arakawa A, Tabata T, Toyoda S. Phase II study of carboplatin and weekly irinotecan combination chemotherapy in recurrent ovarian cancer: a Kansai clinical oncology group study (KCOG0330). Anticancer Res 2013; 33: 1073-1079.

10) Giessen C, Laubender RP, Fischer von Weikersthal L, Schalhorn A, Modest DP, Stintzing S, Haas M, Mansmann UR, Heinemann V. Early tumor shrinkage in metastatic colorectal cancer: retrospective analysis from an irinotecan-based randomized first-line trial. Cancer Sci 2013; 104: 718-724.

11) Abu Lila AS, Nawata K, Shimizu T, Ishida T, Kiwada H. Use of polyglycerol (PG), instead of polyethylene glycol (PEG), prevents induction of the accelerated blood clearance phenomenon against long-circulating liposomes upon repeated administration. Int J Pharm 2013; 456: 235-242.

12) Tyagi N, Ghosh PC. Folate receptor mediated targeted delivery of ricin entrapped into sterically stabilized liposomes to human epidermoid carcinoma (KB) cells: effect of monensin intercalated into folate-tagged liposomes. Eur J Pharm Sci 2011; 43: 343-353.

13) Abu-Lila A, Suzuki T, Doi Y, Ishida T, Kiwada H. Oxaliplatin targeting to angiogenic vessels by PEGylated cationic liposomes suppresses the angiogenesis in a dorsal air sac mouse model. J Control Release 2009; 134: 18-25.

14) Sonoke S, Ueda T, Fujiwara K, Sato Y, Takagaki K, Hirabayashi K, Ohgi T, Yano J. Tumor regression in mice by delivery of Bcl-2 small interfering RNA with pegylated cationic liposomes. Cancer Res 2008; 68: 8843-8851.

15) Ho EA, Osooly M, Strutt D, Masin D, Yang Y, Yan H, Bally M. Characterization of long-circulating cationic nanoparticle formulations consisting of a two-stage PEGylation step for the delivery of siRNA in a breast cancer tumor model. J Pharm Sci 2013; 102: 227-236.

16) Nie Y, Ji L, Ding H, Xie L, Li L, He B, Wu Y, Gu Z. Cholesterol derivatives based charged liposomes for doxorubicin delivery: preparation, *in vitro* and *in vivo* characterization. Theranostics 2012; 2: 1092-1103.

17) Abu Lila AS, Okada T, Doi Y, Ichihara M, Ishida T, Kiwada H. Combination therapy with metronomic S-1 dosing and oxaliplatin-containing PEG-coated cationic liposomes in a murine colorectal tumor model: synergy or antagonism. Int J Pharm 2012; 426: 263-270.

18) Di Donato L, Cataldo M, Stano P, Massa R, Ramundo-Orlando A. Permeability changes of cationic liposomes loaded with carbonic anhydrase induced by millimeter waves radiation. Radiat Res 2012; 178: 437-446.

19) Lee SM, Ahn RW, Chen F, Fought AJ, O'Halloran TV, Cryns VL, Nguyen ST. Biological evaluation of pH-responsive polymer-caged nanobins for breast cancer therapy. ACS Nano 2010; 4: 4971-4978.

20) Lee SM, Lee OS, O'Halloran TV, Schatz GC, Nguyen ST. Triggered release of pharmacophores from [Ni($HAsO_3$)]-loaded polymer-caged nanobin enhances pro-apoptotic activity: a combined experimental and theoretical study. ACS Nano 2011; 5: 3961-3969.

21) He F, Deng X, Wen B, Liu Y, Sun X, Xing L, Minami A, Huang Y, Chen Q, Zanzonico PB, Ling CC, Li GC. Noninvasive molecular imaging of hypoxia in human xenografts: comparing hypoxia-induced gene expression with endogenous and exogenous hypoxia markers. Cancer Res 2008; 68: 8597-8606.

22) Xu M, Wang G, Xie H, Huang Q, Wang W, Jia Y. Pharmacokinetic comparisons of schizandrin after oral administration of schizandrin monomer, Fructus Schisandrae aqueous extract and Sheng-Mai-San to rats. J Ethnopharmacol 2008; 115: 483-488.

23) Lee HJ, Paik WH, Lee MG. Pharmacokinetic and tissue distribution changes of adriamycin and adriamycinol after intravenous administration of adriamycin to alloxan-induced diabetes mellitus rats. Res Commun Mol Pathol Pharmacol 1995; 89: 165-178.

24) Nagarwal RC, Kant S, Singh PN, Maiti P, Pandit JK. Polymeric nanoparticulate system: a potential ap-

proach for ocular drug delivery. J Control Release 2009; 136:2-13.

25) Cuong NV, Hsieh MF. Molecular targeting of liposomal nano-particles to lymphatic system. Curr Cancer Drug Targets 2011; 11: 147-155.

26) Allen T M, Cullis PR. Drug Delivery Systems: Entering the Mainstream. Science 2004; 303: 1818-1822.

27) Peer D, Karp JM, Hong S, Farokhzad OC, Margalit R, Langer R. Nanocarriers as an emerging platform for cancer therapy. Nat Nanotechnol 2007; 2: 751-760.

28) Cuomo F, Mosca M, Murgia S, Avino P, Ceglie A, Lopez F. Evidence for the role of hydrophobic forces on the interactions of nucleotide-monophosphates with cationic liposomes. J Colloid Interface Sci 2013; 410: 146-151.

29) Barichello JM, Kizuki S, Tagami T, Soares LA, Ishida T, Kikuchi H, Kiwada H. Agitation during lipoplex formation harmonizes the interaction of siRNA to cationic liposomes. Int J Pharm 2012; 430: 359-365.

30) Banerjee A, Roychoudhury J, Ali N. Stearylamine-bearing cationic liposomes kill Leishmania parasites through surface exposed negatively charged phosphatidylserine. J Antimicrob Chemother 2008; 61: 103-110.

31) Kontek R, Matlawska-Wasowska K, Kalinowska-Lis U, Marciniak B. Genotoxic effects of irinotecan combined with the novel platinum(II) complexes in human cancer cells. Chem Biol Interact 2010; 188: 66-74.

32) Bothun GD, Lelis A, Chen Y, Scully K, Anderson LE, Stoner MA. Multicomponent folate-targeted magnetoliposomes: design, characterization, and cellular uptake. Nanomedicine 2011; 7: 797-805.

33) Kim JY, Kim JK, Park JS, Byun Y, Kim CK. The use of PEGylated liposomes to prolong circulation lifetimes of tissue plasminogen activator. Biomaterials 2009; 30: 5751-5756.

34) Mager DE, Mody V, Xu C, Forrest A, Lesniak WG, Nigavekar SS, Kariapper MT, Minc L, Khan MK, Balogh LP. Physiologically based pharmacokinetic model for composite nanodevices: effect of charge and size on *in vivo* disposition. Pharm Res 2012; 29: 2534-2542.

35) Cheng SH, Li FC, Souris JS, Yang CS, Tseng FG, Lee HS, Chen CT, Dong CY, Lo LW. Visualizing dynamics of sub-hepatic distribution of nanoparticles using intravital multiphoton fluorescence microscopy. ACS Nano 2012; 6: 4122-4131.